Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

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Pertanika Journal of Tropical Agricultural Science
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About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science is an official journal of Universiti Putra Malaysia. It is an open-access online scientific journal. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognised internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields.

Pertanika Journal of Tropical Agricultural Science is a **quarterly** (*February*, *May*, *August*, and *November*) periodical that considers for publication original articles as per its scope. The journal publishes in **English** and it is open for submission by authors from all over the world.

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Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance, and veterinary medicine.

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Pertanika was founded in 1978. A decision was made in 1992 to streamline *Pertanika* into 3 journals as Pertanika Journal of Tropical Agricultural Science, Pertanika Journal of Science & Technology, and Pertanika Journal of Social Sciences & Humanities to meet the need for specialised journals in areas of study aligned with the interdisciplinary strengths of the university.

Currently, as an interdisciplinary journal of agriculture, the revamped journal, a leading agricultural journal in Malaysia now focuses on tropical agricultural research and its related fields.

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To publish journals of international repute.

Mission

Our goal is to bring the highest quality research to the widest possible audience.

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We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 90 days. The elapsed time from submission to publication for the articles averages 180 days. We are working towards decreasing the processing time with the help of our editors and the reviewers.

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Pertanika is now over 42 years old; this accumulated knowledge has resulted in Pertanika Journal of Tropical Agricultural Science being abstracted and indexed in SCOPUS (Elsevier), Clarivate Web of Science (ESCI), EBSCO, DOAJ, Agricola, ASEAN CITATION INDEX, ISC, Microsoft Academic, Google Scholar, National Agricultural Science (NAL), and MyCite.

Citing journal articles

The abbreviation for Pertanika Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci*.

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An ISSN is an 8-digit code used to identify periodicals such as journals of all kinds and on all media–print and electronic. All *Pertanika* journals have an e-ISSN.

Pertanika Journal of Tropical Agricultural Science: e-ISSN 2231-8542 (Online).

Lag time

A decision on acceptance or rejection of a manuscript is expected within 90 days (average). The elapsed time from submission to publication for the articles averages 180 days.

Authorship

Authors are not permitted to add or remove any names from the authorship provided at the time of initial submission without the consent of the journal's Chief Executive Editor.

Manuscript preparation

Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words ***I****ntroduction*, ***M****aterials and Methods*, ***R****esults*, ***A****nd* ***D****iscussion*. IMRAD is simply a more 'defined' version of the "IBC" (*Introduction*, *Body*, *Conclusion*) format used for all academic writing. IMRAD indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: *Title*, *Authors*, *Keywords*, *Abstract*, *Conclusions*, *References*, and *Acknowledgement*. Additionally, some papers include *Appendices*.

The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the journal's **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_ Authors.pdf).

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Notification of the editorial decision is usually provided within 90 days from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

As articles are double-blind reviewed, material that may identify authorship of the paper should be placed only on page 2 as described in the first-4-page format in *Pertanika*'s **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_Authors.pdf).

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In the peer review process, 2 or 3 referees independently evaluate the scientific quality of the submitted manuscripts. At least 2 referee reports are required to help make a decision.

Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

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What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are 7 steps to the editorial review process:

1. The journal's Chief Executive Editor and the Editor-in-Chief examine the paper to determine whether it is relevance to journal needs in terms of novelty, impact, design, procedure, language as well as presentation and allow it to proceed to the reviewing process. If not appropriate, the manuscript is rejected outright and the author is informed.

2. The Chief Executive Editor sends the article-identifying information having been removed, to 2 or 3 reviewers. They are specialists in the subject matter of the article. The Chief Executive Editor requests that they complete the review within 3 weeks.

   Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the research field.

3. The Editor-in-Chief examines the review reports and decides whether to accept or reject the manuscript, invite the authors to revise and resubmit the manuscript, or seek additional review reports. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the authors) are forwarded to the authors. If a revision is indicated, the editor provides guidelines to the authors for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the Chief Executive Editor along with specific information describing how they have answered' the concerns of the reviewers and the editor, usually in a tabular form. The authors may also submit a rebuttal if there is a need especially when the authors disagree with certain comments provided by reviewers.

5. The Chief Executive Editor sends the revised manuscript out for re-review. Typically, at least 1 of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the Editor-in-Chief examines their comments and decides whether the manuscript is ready to be published, needs another round of revisions, or should be rejected. If the decision is to accept, the Chief Executive Editor is notified.

7. The Chief Executive Editor reserves the final right to accept or reject any material for publication, if the processing of a particular manuscript is deemed not to be in compliance with the S.O.P. of *Pertanika.* An acceptance notification is sent to all the authors.

   The editorial office ensures that the manuscript adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any minor queries by the editorial office. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, **only essential changes are accepted**. Finally, the manuscript appears in the pages of the journal and is posted on-line.

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ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

The journal is indexed in SCOPUS (Elsevier), Clarivate-Emerging Sources Citation Index (ESCI), BIOSIS, National Agricultural Science (NAL), Google Scholar, MyCite, ISC. In addition, Pertanika JSSH is recipient of "CREAM" Award conferred by Ministry of Higher Education (MoHE), Malaysia.

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Contents

Foreword

Welcome to the third issue of 2022 for the Pertanika Journal of Tropical Agricultural Science (PJTAS)!

PJTAS is an open-access journal for studies in Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university for the benefit of the world-wide science community.

This issue contains 18 articles; four review articles, one short communication, one case study and the rest are regular articles. The authors of these articles come from different countries namely Bangladesh, Indonesia, Malaysia and Thailand.

A selected article entitled "Feed Intake and Apparent Nutrient Digestibility of Growing Rabbits Fed *Asystasia gangetica* with Different Levels of Corn" identified the effect of different corn levels on intake and digestibility in rabbits. The rabbits were divided into four groups and given *Asystasia gangetica ad libitum* as a basal diet and supplemented with either 80 g (T1), 60 g (T2), 40 g (T3), or 0 g (T4) corn/head/day. This study concluded that the apparent nutrient digestibility was significantly improved in growing rabbits following supplemental feeding with a diet containing 60-80 g of corn. The further details of this study are found on page 587.

A regular article entitled "Alternate Wetting and Drying (AWD) on Rice Irrigation" evaluated the feasibility of alternate wetting and drying (AWD) implementation by applying two treatments: continuously flooded (control) and AWD irrigation, onto the paddy cultivation. The growth performance evaluation, the grain yield performance evaluation, and the chlorophyll measurement were done. However, the analysis showed that there is no significant difference at $p < 0.05$, a 95% confidence level. The detailed information of this article is available on page 649.

Suharjo and Suaib from Indonesia investigated the effects of planting media and gutter slopes on the growth of lettuce (*Lactuca sativa* L.) using the Nutrient Film Technique (NFT) in a hydroponic system. There are two types of treatment, which involved the differences in media and the gutter slope of pipes, respectively. A randomized block design (RBD) with three replications was carried out. The plant height, number of leaves,

and plant fresh weight were the observed variables, and analyzed using analysis of variance (ANOVA) followed by the least significant difference (LSD) test at a 5% level. The results revealed that the treatments had significantly affected the growth of the lettuce. Full information of this study is presented on page 805.

We anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

All the papers published in this edition underwent Pertanika's stringent peer-review process involving a minimum of two reviewers comprising internal as well as external referees. This was to ensure that the quality of the papers justified the high ranking of the journal, which is renowned as a heavily-cited journal not only by authors and researchers in Malaysia but by those in other countries around the world as well.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers, Editor-in-Chief and Editorial Board Members of PJTAS, who have made this issue possible.

PJTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

Chief Executive Editor
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Do it Yourself: Humic Acid

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ABSTRACT

The humic substance consists of humic acid, fulvic acid, and humin. Humic acid is a useful metal complexing agent, a good dispersant, and a redox agent. Humic acid showed an auxin-like activity and thus promoted root growth and development. It positively affected soil's physical, chemical, and biological properties. Hence, humic acid indirectly improved plant growth by chelating nutrients to the plant. However, humic acid converted carcinogen compounds in chlorinated water. Still, humic acid is a good compound for agricultural purposes. Humic acid can be produced in thermophilic composting, vermicomposting, and Bokashi. The humification process can occur with decomposers such as black soldier fly. Those methods can be made in farmland and even in the housing area. Extraction of humic acid is required from those production methods. However, it is not easy to extract by farmers on a small scale. Full compost and Bokashi or its tea also showed much humic acid alone. Humic acid extraction may be optional but good as crop tonic. Nonetheless, further study should be carried out. Bokashi tea and leachate with decomposer should be further studied to obtain more evidence of their benefits. With the benefit of composting and fermentation, further study on treating is required for food security.

Keywords: Bokashi, compost, humic acid, soil amendment, vermicompost

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INTRODUCTION

Characteristics of Humic Acid

Humic substances are an important component of the earth, especially soil organic matter. They are vital for soil's physical and chemical processes (Gautam et al., 2021). Humic substances consist of at least half chemical-resistant organic carbon

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(Gautam et al., 2021; Hayes et al., 1989; Lal, 2004). The humification process is mainly contributed by plant and microbial biomass (Gautam et al., 2021). Humic substances are not easily decomposed or degraded and are used by microbes due to the complex compounds forming cell membranes and walls (Gautam et al., 2021; Kögel-Knabner, 2002). They provide brown-black color to soils but depend on composting raw materials and their concentration (Gautam et al., 2021).

Humic substances are formed in the mixture of humic acids (insoluble below pH 2), fulvic acids (soluble at any pH), and humin (insoluble in water) (Figure 1) (Frimmel & Christman, 1988; Gautam et al., 2021). They are a valuable metal complexing agent, good dispersant, and redox agent (Schnitzer, 1991). In a strong base, the extract is acidified, and the complex macromolecular organic substance of humic acid is coagulated, whereas fulvic acid remains soluble (Bleam, 2017; Schnitzer, 1991). As pH is reduced, humic acid, a weak acidic electrolyte, turns hydrophobic (Singh, 2015) and decreases negative charge (Kinniburgh et al., 1998). It is a redox-active compound in soil and the aquatic system. Humic acid comprises a heterogeneous mixture of polymerized acids (not easily separated and analyzed) and phenolic radicals in a state of advanced decay.

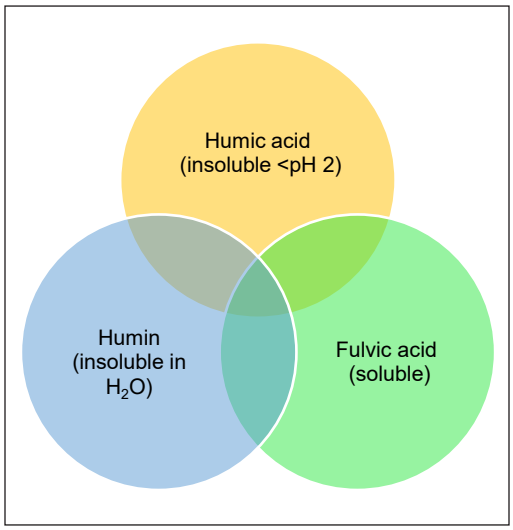

Figure 1. The humic substance consists of humic acid, fulvic acid, and humin

Humic acid can naturally occur in nature. It is found in the intermediate state after passing through the stages of compost and peat and before becoming lignite. Lignite coals are used in developed countries as a power supply source, whereas developing countries use them as agricultural humic acid-rich products (Susic, 2016). Therefore, they are important as the precursors of peat, asphalt, kerogens, petroleum, bitumen, and coal (Achard, 1786). The pathways and rate of a redox reaction are depended on the solution chemistry, material type, and microbial activity.

Even though the input of many biodegradable materials via composting, the percentage of extractable humic acid was low. Composting biodegradable materials via thermophilic compost, vermicompost, and Bokashi produced different percentages of extractable humic acid, from 8.4±1.1% to 22.9±2.6% (Epelde et al., 2018). The humic acid to fulvic acid ratio is generally high in most composting methods (Epelde et al., 2018). Humic acid will obtain very slowly if not composted. However, composting can be done easily without extra care and cost.

Effect of Humic Acid on Soil

Humic substances affect the nature and content of soil organic matter. Hence, it has an important role in the soil structure and function (Khaled & Fawy, 2011; Yang et al., 2004b). Furthermore, humic acid enhances soil nutrient availability and soil water holding capacity (Khaled & Fawy, 2011). Therefore, humic acid extracted from pulverized weathered coal was suitable to apply in the field since it decreased the ammonia (NH_3) volatilization and carbon dioxide (CO_2) emissions (Pang et al., 2021). In addition, humic acid significantly decreased water evaporation and enhanced water use efficiency by plants in low clay content and water holding capacity soils (e.g., sandy and arid soils) (Khaled & Fawy, 2011).

Humic acid significantly improved soil macroaggregates and could efficiently reduce soil salinity (M. Liu et al., 2019). By boosting up the cohesive forces of very fine soil particles (<0.002 mm), humate reduced soil erosion (Khaled & Fawy, 2011). Humic acid could affect the soil microbe community; for instance, fungal and bacterial community structures in the different growth stages were enhanced soil critical nutrient level and plant nutrient sufficiency range (M. Liu et al., 2019), where humic acid promoted nutrient chelate and made nutrients available to plants (Khaled & Fawy, 2011). Soil urease and sucrase activity enhanced after applying humic acid (Pang et al., 2021).

Furthermore, humic acid had the potential to act as a washing agent for the soil's toxic elements, including arsenic (As), cadmium (Cd), manganese (Mn), nickel (Ni), lead (Pb), and zinc (Zn) in the *in-* or *ex-situ* remediation mechanism (Mosa et al., 2021). Biochar-humic acid-wood vinegar acted as a radiation material to immobilize nickel (Ni) metal in soil effectively (Zhu et al., 2021). In addition, the role of wood vinegar was to increase the oxygen-containing group (Zhu et al., 2021). Humic acid is extracted from fast-growing plants, such as corn (16 days). Stover anaerobic digestion had higher heavy metal adsorption ability for copper(II) ion (Cu(II)), cobalt(II) ion (Co(II)), and nickel(II) ion (Ni(II)) than livestock manure anaerobic digestion, such as chicken (X. Wang et al., 2021b). Perhaps, the co-remediation of humic acid and phytoremediator can be increased the remediate power in the polluted area, including the factory area.

In short, humic acid can improve soil properties and microbial activity and remediate contaminated soil.

Effect of Humic Acid on Plant

Humic acid is important for enhancing plant growth and development, nutrient and water retention, and suppressing diseases (Guo et al., 2019). Humic acid showed an auxin-like activity (Bottomley, 1917; Canellas et al., 2002) and the presence of interchangeable auxin groups in the macrostructure (Canellas et al., 2002). Humic acid accelerates cell division (Khaled & Fawy, 2011), promotes lateral root development, increases root surface area (Malik & Azam, 1985; Schnitzer, 1991), and decreases stress deterioration (Khaled

& Fawy, 2011) with the presence of auxin group in humic acid. Its size-fractions altered the root structure and thus affected root growth patterns, including providing more branched roots, size, and hairs to increase surface area (Canellas et al., 2010).

Humid acid promotes the expression of proton pump (H^+-ATPase) (Canellas et al., 2002). Auxin stimulates plant growth by increasing the amount of plasma membrane H^+-ATPase, acidifies the apoplast, and thus slackens the cell wall, promoting cell elongation (Frías et al., 1996; Hager et al., 1991). The increase in root hair enhanced the nutrient uptake and activation of the H^+-ATPase. It improves plant nutrition by improving the electrochemical proton gradient that makes ion transport across the cell membrane through secondary transport systems.

In a recent study, alkamides, secondary metabolites, were found in humic acid and positively affect root growth, similar to auxin (Zandonadi et al., 2019). Alkamides alter particular root signal transduction cascades that change root growth, but the differentiation is still unknown (Morquecho-Contreras et al., 2010). It was confirmed that the involvement of cytokinin receptors and nitric oxide production during root development (López-Bucio et al., 2007). Many Gram-negative bacteria produce alkamide-related substances such as N-acyl-L-homoserine lactones. The plant received N-acyl-L-homoserine lactones to alter root architecture and senescence-related processes by intermingling with jasmonic acid signaling (Morquecho-Contreras et al., 2010).

Root growth of marigold, strawberry, and pepper significantly increased by substituting 250 to 1,000 mg humates kg^{-1} dry weight of commercial soilless potting medium (Metro-Mix®360, Sun Gro Horticulture, USA) (Arancon et al., 2006). In addition, the co-application of 10 mM K and 2 h kg^{-1} humic acid allow 100 mM sodium chloride (NaCl) salt-stressed salt-sensitive wheat plant root to grow (Abbas et al., 2022).

Humic acid also showed positive effects on the growth of beneficial microbes in the compost. For example, humic acid-enriched vermicompost improves arbuscular mycorrhizal fungi colonization, root nodulation, and plant growth maximally and eventually improves plant growth and soil health (Maji et al., 2017). In addition, the application of vermicompost suppresses soil-borne plant pathogen of mung bean (Saxena et al., 2015).

Humic acid can enhance the corn yield in salinized soil by enhancing nutrient absorption in different growth stages (M. Liu et al., 2019). Nitrogen (N) nutrient absorption in the corn vegetative growth period and phosphorus (P) and potassium (K) nutrient absorption in the reproductive growth period, especially the tasselling and harvest stage, was improved with the presence of humic acid (M. Liu et al., 2019).

Moreover, applying humic acid to plants allows the plant to cope with environmental stress. For example, the co-application of K and humic acid allows salt-stressed salt-tolerant wheat to shoot dry matter and nutrients (K, Fe, Zn) accumulated as

non-stressed ones (Abbas et al., 2022). In addition, the co-application of K and humic acid reduced the Na accumulation in salt-stressed wheat plants significantly in both root and shoot (Abbas et al., 2022).

Humic acid application (54 mg L^{-1}) allowed 22% of seedling N uptake and shoot dry matter improved (Malik & Azam, 1985). Humic acid-enhanced N accumulation and thus improved chlorophyll content in the plant; however, the concentration of the humic substance is the matter (Malik & Azam, 1985; Xu et al., 2012). Also, humic acid application significantly increased the soil plant analysis development (SPAD) reading of the snap bean plant (El Sheikha et al., 2022). In addition, humic acids relieve adjusting plant chlorosis, strengthen plant enzyme systems, and enhance the permeability of the plant membranes (Khaled & Fawy, 2011).

Fulvic acid significantly promotes high chlorophyllase *a* activity than either humic acid or humin (Yang et al., 2004b). Humic acid significantly promotes the activity of chlorophyllase *b* than either humin or fulvic acid (Yang et al., 2004b). However, humin does not significantly affect the activity of chlorophyllase *a* and *b* (Yang et al., 2004a). Some of the phenolic acids, the important component of humic substances (Cheshire et al., 1967), such as *p*-coumaric acid, ferulic, and *o*-hydroxyphenyl acetic, showed an antagonistic effect on Mg-chelatase, the chlorophyll biosynthetic enzyme (Yang et al., 2002). Nonetheless, those compounds synergistically affected the activities of Mg-dechelatase *a* and *b* and chlorophyllase *a* and *b* in paddy seedlings (Yang et al., 2004a). Therefore, other factors also affected the chlorophyllase activity (Yang et al., 2004b).

Humid acid-enhanced our taste buds and economic value to selling the crop. For instance, total soluble solids, ascorbic acid content, dry matter contents, fruit characteristics (e.g., fruit height, diameter, weight, and fruit number per plant), and the yield of tomatoes have remarkably improved by humic acid (Yildirim, 2007). Also, humic acid has significantly improved the number of flowers and fruits (Arancon et al., 2006). Furthermore, it can resist plant disease (Khaled & Fawy, 2011) and thus improve food and nutrient security. The humic acid applied to time for fruit and vegetable crops can be further studied in the future and thus ensure the plant has fully utilized the resource.

Salicylic acid and humic material could act as a cut flower postharvest preservative, where they decrease the lipid peroxidation and delay the aging process (Khandan-Mirkohi et al., 2021). In addition, the activity of the antioxidant enzymes is enhanced by salicylic acid and humic material in cut flowers (Khandan-Mirkohi et al., 2021). Perhaps, this concept can be further applied in stem cutting, selling, and big tree transplanting (to preserve the root ball) to extend the shelf life for shipping and storage time.

Humic acid has many positive effects on plant growth and development, even in the postharvest stage. However, the effect of humic acid is highly dependent on the nature of humic substances, including

composition, concentration, and pH (e.g., growth medium, culture condition, and plant species) (Schnitzer, 1991).

Effect of Humic Acid on Water Bodies

Modified humic acid significantly remediated the water bodies from heavy metals. Humic acid had superior heavy metal (e.g., Cd, Cu, Pb, and Zn) wash out capacity than artificial sweeteners such as acesulfame and sucralose in lake sediment remediation (Y. Liu et al., 2020b). In addition, the low adsorption capacity of humic acid had enhanced the affinity of magnetic nanoparticles with oxidizing and ferrous ferric oxide (Fe_3O_4) coating to treat drinking water (Xue et al., 2021).

Nevertheless, humic acid brings many benefits to agriculture and the environment. On the flip side, humic acid is recommended to remove from water for drinking and swimming water use purposes. Humic acid is converted to carcinogenic compounds such as trihalomethanes and haloacetic acids after chlorinating in drinking and swimming water (Satheesh Ananda & Mehendale, 2005; Shi et al., 2020).

Humic molecular are vulnerable to being attacked by chlorine as they are electron-rich with aliphatic side chains and phenolic structures (Liao et al., 1982; Richard, 1982). Therefore, they have the potential as the precursor's toxic disinfection by-products with the carbon attached (Rook, 1976). The by-products can be classified into volatile hydrophobic and non-volatile hydrophilic. The specific by-product of humic acid depends on its molecular structure, pH, and chlorine to carbon ratio (Richard, 1982). High chlorine to carbon ratio can produce a non-volatile hydrophilic humic acid by-product (Richard, 1982; Rook, 1977). Humic acid changes the unchlorinated watercolor and affects the aesthetic value.

Spot of Humic Acid

Humic acid is an organic matter found in the sediment, terrestrial soil, and natural water (de Melo et al., 2016). Also, humic acid can be formed through human activity such as thermophilic composting, vermicomposting, and fermenting. Therefore, it is easy to be made by ourselves. Various ways can form the humus substance, such as thermophilic composting, vermicomposting, fermenting (Bokashi), and even decomposer (i.e., black soldier fly). The concentration of humic acid in different recipes of thermophilic compost (e.g., chicken manure-based or green waste thermophilic compost) and vermicompost (e.g., fresh, aged chicken manure-based or food waste vermicompost) is in the range of 2.9 to 11.5 g kg^{-1} dry weight (Pant et al., 2012b).

Thermophilic Compost. Compost generally undergoes mesophilic, thermophilic, and stable phases. Among the phases, the thermophilic phase has the peak temperature, from 45 °C up to 70 °C; however, there is no specific temperature (Miller, 1996). During the transition phase from mesophilic to thermophilic, the microbial activity will be reduced (Day et al., 1998). Thermophilic compost is a product that undergoes the process to achieve a sanitary and stable

compost with a controlled biological decomposition of biodegradable materials such as agricultural and kitchen waste, mainly under aerobic conditions, and allows the development of thermophilic temperatures biologically (Walters, 2009).

A good quality compost has moist, fine-textured, pathogen-free, and comprises high beneficial microbes, soluble mineral nutrients, phytohormones, humic substances, and low phytotoxic organic acids and heavy metals. Humic substances can be used as an indicator of the maturity of compost (Wei et al., 2007). Manipal solid waste compost had significantly higher extractable humic acid (22.7±1.8%) than composts (Epelde et al., 2018). Compost tea is the water extract from compost substrate soaked with recirculated water in aerobic conditions (Riggle, 1996). Therefore, the compost tea quality depended on the compost quality (Milinković et al., 2019; Naidu et al., 2013; Pant et al., 2012b). Compost and manure are widely used for plant disease management, whereas compost tea has recently been used as a crop tonic (Walters, 2009).

Humification also allows lignin degradation (Burges et al., 1964; Steelink, 1964). Lignocellulose degradation improved by adding humic acid-modified oyster shell to the digestate (obtained through aerobic fermentation) composting; however, the synthesis of nitrate (NO_3^-) decreased (Lu et al., 2021). Humic substances formed from red mud composting promote lignin degradation and laccase-producing microbes' growth (Jiang et al., 2021). The addition of manganese dioxide (MnO_2) fastened the organic matter degradation and enhanced the humification degree of fulvic acid in the co-composting of chicken manure and rice straw (Qi et al., 2021).

Animal-based manure thermophilic compost significantly suppressed disease in livestock production and controlled powdery mildew disease in crop production than plant-based thermophilic compost (Pant et al., 2012b; Weltzien, 1990). In contrast, green waste thermophilic compost and its tea showed significantly high humic acid than manure-based compost (Pant et al., 2012b). In addition, the co-application of N fertilizer, humic acid, and plant-based (rice straw) compost significantly enhanced the wheat growth performance and yield (Antoun et al., 2010).

Vermicompost. Vermicompost is the product of speeding up the biodegradation of organic matter, such as agricultural, industrial, and urban waste, using fauna such as earthworms without a thermophilic stage (Hervas et al., 1989). Up to 50% of the organic fraction total weight in the vermicompost are sterols, proteins, polysaccharides, fatty acids, alkanes, and enzymes; however, humic acid was in the range of 4–17% of the total weight (Epelde et al., 2018; Hervas et al., 1989).

Vermicompost has higher phosphorous (P), potassium (K), magnesium (Mg), sulfur (S), calcium (Ca), and carbon (C) than thermophilic compost (Hervas et al., 1989; Tognetti et al., 2005). Due to the presence of phytohormone, auxin, in humic acid, flowering and fruiting significantly improved in the food waste vermicompost humic acid

treated crop compared to commercial humic acid (Arancon et al., 2006). Furthermore, the application of humic acid (100 mg L^{-1}) significantly improved plant morphology (leaf area, length, and branch diameter) and yield (cluster weight, number of fruits per cluster, and fruit weight) (Pakkish et al., 2022). In addition, vermicompost and humic acid significantly improved secondary plant metabolites (e.g., flavonoid and phenolic) (Gholami et al., 2018) which is good for food and nutrient security.

Compost and vermicompost tea can be produced by brewing with water. The dilution from 1:10 to 1:20 can obtain optimum plant growth (Pant et al., 2012a, 2012b). The tea and humic acid positively affected plant growth, yield, quality, and nutrition (El Sheikha et al., 2022). Besides, vermicompost tea can be produced by leaching the solution during the composting process, which also contains micronutrients, fulvic acid, and humic acid to improve plant growth (Gutiérrez-Miceli et al., 2008). Vermicompost tea might be allowed insignificant changes in transamination and deamination reactions in the amino acid profiles (El Sheikha et al., 2022). Vermicompost tea contained more humic acid than vermicompost (Pant et al., 2012a). Vermicompost leachate also has the potential to substitute for P and K deficiency (Arthur et al., 2012).

Bokashi. Fermentation is an anaerobic process with microbes to form peat where the breakdown of plant remains is terminated by bathing in water (H_2O) with a very low oxygen (O_2) content plus the presence of various organic acids, including humic acid (Merfield, 2012). Bokashi is one example of organic matter fermentation. Bokashi is fermented organic matter (e.g., cooked and raw plant and animal-based materials) made by fermenting with effective microorganisms, molasses, and water for about two weeks and sun-dried within two days (to avoid secondary fermentation) (Christel, 2017). The receipt is versatile to change with the preference of raw materials. It brings many benefits to plant growth.

Bokashi improved seedling survival rate and height during transplanting (Jaramillo-López et al., 2015) and showed the greatest positive effect on initial plant growth and development (Bócoli et al., 2020). In addition, humic acids extracted from Bokashi demonstrated positive effects on the initial corn growth performance and development (Baldotto & Baldotto, 2016) and chlorophyll content (Prisa, 2020; Santos et al., 2020), and photosynthetic capacity (Olle, 2021). Amendment of Bokashi enhanced plant height of *Alibertia edulis* (Santos et al., 2020), onion (Álvarez-Solís et al., 2016), and jalapeño pepper plants (Álvarez-Solís et al., 2016).

Humic acid extracted from Bokashi tea significantly increased dry matter accumulation up to 41% at 45.70 mmol/L humic acids compared to control (Baldotto & Baldotto, 2016). Bokashi had significantly low extractable humic acid (8.4±1.1%) than composts (Epelde et al., 2018). Tomato seed soaking with Bokashi tea enlarged transplants' stem diameter, promoting plant nutrient absorption (Olle, 2020). Bokashi leachate as a seed priming agent

also positively affects seed germination and dormancy breaking (Phooi et al., in press).

Black Soldier Fly Larvae

The decomposer of the black soldier fly (*Hermetia illucens* L.) could allow the easy biodegradable organic matter, including soluble microbial by-products and proteinaceous substances, to convert to humic substances (Q. Wang et al., 2021a). Raw livestock manure such as chicken, cow, and pig manure as black soldier fly larvae feed can increase the humic-like and fulvic-like substance (Q. Wang et al., 2021a). It can alter the structure and composition of animal manure (e.g., cow, chicken, and pig) (Q. Wang et al., 2021a). As a bonus, it also makes the manure more aroma due to the aromatic protein formation (Q. Wang et al., 2021a). It can be used as a post-composting agent to boost the level of the humic substance. It can improve the maturity of compost (T. Liu et al., 2020a).

Black soldier fly is known as environment cleaner. It can clean the organic waste and reduce the spread of disease (Bradley & Sheppard, 1984). Biotransformation from black soldier fly larvae can suppress pathogens such as *Salmonella* and *Escherichia coli* in livestock manure, control horsefly breeding, and reduce manure waste (Erickson et al., 2004; Q. Li et al., 2011). *Escherichia coli* count in the diary manure was significantly reduced by black soldier fly at the constant temperature of 27 °C (Q. Liu et al., 2008). Copper (Cu) and zinc (Zn) mobilization can be improved with the presence of black soldier fly larvae (T. Liu et al., 2020a) and thus positively affect plant growth.

Extraction Method of Humic Acid

Humic acid extraction needs alkaline extraction, humic acid separation, and fulvic acid separation (Lamar et al., 2014). The sample should be crushed and sieved with 60 mesh.

The following extraction method was described by (Canellas et al., 2002; Schnitzer, 2015). A 1:10 ratio (v/v) of sample and 0.5 M sodium hydroxide (NaOH) needed to mix under nitrogen (N_2) atmosphere for 12 hours. First, the suspension was centrifuged at 5,000 × *g* and then acidified to pH 1.5 using 6 M hydrochloric acid (HCl). The third solubilized and precipitated humid acid pallet was then mixed with 10 volumes of a diluted mixture of hydrofluoric acid (HF) and HCl solution (5 mL L^{-1} of 12 M HCl + 5 mL L^{-1} of 48%, v/v HF). A negative test against silver nitrate ($AgNO_3$) was obtained by repeatedly washing with H_2O after centrifugation at 5,000 × *g* for 15 minutes. Then, purifying against deionized H_2O using a 12-14 kD cut-off membrane is required. Next, the dialysate was lyophilized to form a powder and characterized chemically. Then, the humid acid powder was solubilized with 50 to 100 mL of 0.05 M NaOH, and the pH was adjusted to 5.5 with 0.1 M HCl.

Humic acid extraction was a long process with intensive chemicals and was unsuitable for small-scale extraction. Not only that but the HF and HCl are also required in this method. However, HF and

HCl are highly toxic to human beings and the environment. Moreover, the use of a flame hood is required to protect the experiment conductor, and thus it is impossible to conduct by the small farmer. Besides, humic acid is also extracted through the fungal liquefaction of coal, and also the bioactivity is also improved compared to commercial products (Ghani et al., 2021).

Another alkaline extraction, humic acid, was much simpler. First, the water mixed sample and 1:1 ratio of potassium hydroxide (KOH) and potassium pyrophosphate ($K_4P_2O_7$) were stirred at room temperature. Then, the liquid was separated from the solid to obtain the humic acid extractant (Stevenson, 1994). Still, it was not easily extracted by farmers. Potassium pyrophosphate ($K_4P_2O_7$) is a type of food additive, and thus it is nontoxic to use. However, KOH is very high in pH (base) and harm human if not handled carefully. Therefore, the use of KOH is only recommended in laboratories and industries.

Soil Humus Composition Determination

Soil humus composition determination is generally separating the substances. After applying the humus to soil, humus composition can be analyzed following the method described by (Ndzelu et al., 2021; Zhang et al., 2020). A 5 g of air-dried soil was extracted with 30 mL of 0.1 M alkali solution [NaOH + tetrasodium pyrophosphate ($Na_4P_2O_7$)] under constant shaking at 70 °C for an hour. The mixture was centrifuged for 15 minutes at 548 x *g* and filtered with quantitated fast flow filter paper. The remaining soil residue was humin, and the solution, which was a humic extractable substance, was acidified to pH 1 to separate humic acid from fulvic acid. The C contents of humic extractable, extracted humic acid, and humin are determined using potassium dichromate ($K_2Cr_2O_7$)- sulfuric acid (H_2SO_4) oxidation followed by titration with iron (II) sulfate ($FeSO_4$) (Nelson & Sommers, 1982). Fulvic acid carbon was computed as the difference between humic extractable and humic acid-C. Humification degree was calculated as humic acid-C/ (humic acid-C + fulvic acid-C) × 100.

Nevertheless, it is complicated with the condition (constant shaking temperature) and chemicals needed. Thus, it is impossible to run small-scale farming. Humification can be determined by the naked eye with its color changing.

FUTURE PROSPECT

Compost significantly improved plant growth and slightly altered the rhizosphere microbe community density (e.g., total numbers of bacteria, actinomycetes, and fungi) in tomato plants and thus exhibited antagonistic effects toward the soil-borne root pathogen (de Brito et al., 1995). Compost enhanced cucumber and summer squash (Rashwan et al., 2021). Vermicompost and compost significantly increase aerial and root biomass and plant morphology (e.g., the greater leaves number and leaf area and improved root volume and branching) of the tomato plants (Lazcano et al., 2009). Vermicompost made nutrients available by increasing soil microbial

activity (dehydrogenase activity) and soil respiration, whereas vermicompost tea enhanced soil and foliar nutrient uptake (Pant et al., 2012a).

Bokashi is considered easy to make and fast to get end products among the soil amendments. On the other hand, compost and vermicompost take months to obtain the end product and require basic knowledge to obtain a good compost-like C/N ratio. Also, Bokashi can be Do It Yourself (DIY) with recyclable organic waste such as food waste from family farming, as it is low cost in the production of both the Bokashi and the extraction of humic acid (Baldotto & Baldotto, 2016). However, the study of humic acid extracted from Bokashi, and its leachate is insufficient scientific evidence to prove its benefit to soil and plant growth; however, it is widely used among home gardeners.

Humic acid showed a positive effect on saline soil (Khaled & Fawy, 2011; M. Liu et al., 2019), and hence it may be applied to other infertile soil, such as urban soil and high polluted soil. Urban soil is associated with human being health (G. Li et al., 2018). Urban soil such as Hong Kong, Japan, Germany, the United States of America, the United Kingdom, Russia, and Australia has dumped materials (Jim, 1998; Tiller, 1992). Urban soils have great vertical and spatial variability. For example, each layer may significantly be different in physical properties (e.g., texture, pH, organic matter content, structure, and bulk density) and other associated soil properties, including soil water-holding capacity, aeration, drainage, and fertility. Moreover, urban soil restricts aeration and water drainage in the soil. Also, humic acid had the potential to remediate polluted soil such as factory nearby soil and other anthropogenic soil.

CONCLUSION AND RECOMMENDATIONS

Humic acid can be done yourself by composting, vermicomposting, and Bokashi, as it positively affects soil and plant. Tea with humic acid can be extracted from its compost, brewed with water, or leached. The solids and their tea can be extracted to obtain pure humid acid; however, it is optional since it is similar to tea. Bokashi tea and leachate should be further studied to obtain more evidence of their benefits. With the benefit of composting and fermentation, further study on treating is required for food security.

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Essential Dynamics of Rice Cultivated Under Intensification on Acid Sulfate Soils Ameliorated with Composted Oyster Mushroom Baglog Waste

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ABSTRACT

This study examines the dynamics of essential macro-nutrients for rice cultivation in acid sulfate soils ameliorated with composted oyster mushroom baglog waste. A single factor randomized block design (RBD) was used, and the factors studied include the compost dose of oyster mushroom baglog waste, which consists of 5 treatment levels, namely 0 t ha^{-1} (control), 5 t ha^{-1}, 10 t ha^{-1}, 15 t ha^{-1}, and 20 t ha^{-1}. Furthermore, this study was carried out from May to September 2021 in the rice fields of the Faculty of Agriculture, Lambung Mangkurat University (ULM), Sungai Rangas Village, Banjar Regency, South Kalimantan. The rice plants were cultivated using an intensification technique, and the compost was applied based on the research treatment for two weeks on prepared land before planting. Also, Bartlett's test was carried out before analysis of variance, which had a significant effect of $P<0.05$, and was further tested using Duncan's Multiple Range Test (DMRT) at a 5% level. The results showed variations in the availability of macro-nutrients at five different growth stages: early planting, full vegetative, early panicle emergence, panicle filling, and harvesting phases. The highest levels of ammonium (NH_4^+) and nitrate (NH_3^-) were found in the full vegetative stage, while early planting had the lowest. Also, there was an increase in the available phosphorus (P) from the early planting to the full vegetative stage. The increase in exchangeable potassium (K) occurred at the transition of these stages. These increasing nutrients were due to the addition of the compost. The higher the NH_4^+, NO_3^-, available P, and exchangeable K in acid sulfate soils, the more nitrogen (N), P, and K uptake in rice plants. The

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provision of the compost supplied N, P, and K in available forms and reduced the amount of soluble alumunium (Al) and iron (Fe). Thereby the plant roots absorb the nutrients optimally. Additionally, the compost increased the essential macro-nutrient availability and plant uptake using the rice intensification technique from early planting to harvest.

Keywords: Acid sulfate soils, eco-friendly agriculture, rice intensification, suboptimal land

INTRODUCTION

Rice is the most important food crop in Indonesia and the main raw material for staple foods in most regions. In 2015, the consumption reached 33.3 million tons (Sulaiman et al., 2018). Currently, the largest production centers of this food crop are on the Java and Sumatra islands. During that period, production reached 17.51 million tons throughout the country (Prasetyo et al., 2021). Meanwhile, South Kalimantan could produce up to 0.67 million tons (Prasetyo et al., 2021). According to Statistics Indonesia (Badan Pusat Statistik, BPS) (2020), the population of this province which was 4.3 million, requires up to 0.4 million tons. However, rice demand will increase as the population increases, and predicting current natural conditions tends to be difficult.

Another problem arising is the increasingly limited availability of productive land in the province (Ritung, 2012). Suboptimal lands, such as peat and acid sulfate soils, require proper management when used as arable land (Nursyamsi et al., 2014). Peatland is difficult to manage because of its high environmental risk; hence, it is better to be conserved (Indonesian Agency for Agricultural Research and Development [IAARD], 2011). Meanwhile, acid sulfate soils are productive when managed with the right technology (Saputra & Sari, 2021).

The acid sulfate soils naturally occurred in coastal and inland areas when the sea level rose and immersed the land with sulfate. The sulfate from seawater blends with iron dioxides in the sediments and allows the micro-organisms to establish iron sulfides (FeS_2) under anaerobic conditions (Michael et al., 2015; Sundström et al., 2002). These conditions generate the formation of pyrite (FeS_2), which is the characteristic of acid sulfate soils. Also, land management requires improving soil drainage to provide good aeration for optimal root respiration. However, when the drainage is executed incautiously, the anaerobic condition of the pyrite layer is disturbed. As a result, the sulfide compounds present in the layer will be oxidized to form sulfuric acid and mineral jarosite, which negatively affects plant growth and depreciates macro-nutrients (Michael et al., 2017; Sudarmo, 2004; Sutandi et al., 2011). Another problem with this soil is the low availability of nutrients (Jumar et al., 2021) .

Rice plants need essential nutrients to complete growth and development. Plants that lack these nutrients will fail to germinate and grow roots, stems, leaves, and flowers (Naeem et al., 2017). The essential nutrient consists of macro-nutrients, which

include carbon (C), oxygen (O), hydrogen (H), nitrogen (N), phosphorus (P), and potassium (K). The secondary nutrient consists of calcium (Ca), magnesium (Mg), and sulfur (S), while micro-nutrients consist of chlorine (Cl), iron (Fe), zinc (Zn), manganese (Mn), boron (B), copper (Cu), and molybdenium (Mo) (Etienne et al., 2018). Also, essential macro-nutrients are needed by plants in large quantities; for instance, N is 100 times higher than Zn in plant tissues (Kumar et al., 2021). Rice plants require 165 kg of macro-nutrients, 19 kg of P, and 112 kg of K in one hectare of land (Dobermann & Fairhurst, 2000). In addition to soil chemical properties such as pH, nutrients, and dissolved heavy metals, acid sulfate soils have other problems which lie in their physical and biological properties (Sundström et al., 2002). The technologies to improve the soil conditions and make it productive are amelioration (Saputra & Sari, 2021) and intensification techniques (Upboff, 2008).

The acidic condition of this soil harms productivity and the environment. Therefore, amelioration technology can reduce the impact of soil acidity (Saputra & Sari, 2021). The technology involves the augmentation of substantial materials to affect soil pH and increase nutrient availability (Michael, 2020). Nevertheless, the technique is still dominated by liming input. Although lime is effective for increasing soil pH, it tends to be expensive and unsuitable for large areas. Hence, amelioration using organic materials is recommended to overcome this issue because organic materials are available, feasible to obtain, and environmentally safe. One of these materials is oyster mushroom baglog waste. It is a post-harvest waste obtained from the media during cultivation. The material needs to go through composting to obtain a C/N ratio for macro-nutrients to be easily absorbed by plants and get rid of billions of spore contaminants in the waste (Hunaepi et al., 2018; Susilawati & Raharjo, 2010). Jumar et al. (2020) showed that composted oyster mushroom baglog waste had pH, organic C, total N, carbon to nitrogen (C/N) ratio, total P, and total K of 8.00, 14.38 mg kg^{-1}, 0.74 mg kg^{-1}, 19.43, 0.50%, and 8.08%, respectively, which is in accordance with the appropriate compost quality standard (Indonesian National Standardization Agency [Badan Standarisasi Nasional, BSN], 2004).

Research references globally point to the rice intensification cultivation technique because of its effectiveness on conventional and flooded land. This technique is famous for its rice production technology with high economic value (Hasanah et al., 2021). Furthermore, it emphasizes the management of soil, plant, and water-based on environmentally friendly activities (Upboff, 2008). Razie (2018) reported that rice production using the intensification technique in acid sulfate soils was higher than conventional cultivation with a difference of 0.78 t ha^{-1}. The technique requires a good knowledge of agronomic and environmental management because they are mutually sustainable. The use of organic matter for land amelioration, which is the compost of oyster mushroom

baglog waste, is expected to maximize the potential use of the rice intensification technique in acid sulfate soil. Therefore, this study examines the dynamics of essential macronutrients for rice plants using the intensification cultivation technique on acid sulfate soils ameliorated with compost of oyster mushroom baglog waste.

MATERIALS AND METHODS

This study was carried out for four months, from May to September 2021, in the rice fields of the Faculty of Agriculture, Lambung Mangkurat University (ULM), Sungai Rangas Village, Martapura Barat District, Banjar Regency, South Kalimantan, and the Soil Laboratory, Department of Soil, Faculty of Agriculture, ULM Banjarbaru, South Kalimantan, Indonesia. Furthermore, it was conducted on a 1,000 m^2 rice field of acid sulfate soil. The fields were first cleared of weeds and plowed twice using a hand rotary tractor. Subsequently, a 4 x 4 m size plot was made and separated with a raised bed. The number of plots prepared for the study was 25, divided into five experimental blocks. A single factor randomized block design (RBD) was utilized. Also, the examined factors are the compost dose of oyster mushroom baglog waste which consists of the following five treatment levels, 0 t ha^{-1} (control), 5 t ha^{-1}, 10 t ha^{-1}, 15 t ha^{-1}, and 20 t ha^{-1}. Each treatment had five blocks; hence, 25 experimental units were obtained.

The acid sulfate soil was obtained from Sungai Rangas Village, Banjar Regency, South Kalimantan (3°20'57.5" S 114°46'02.4" E). 'Purun tikus' (*Eleocharis dulcis*) and 'papisangan' (*Ludwigia erecta*) are vegetation that grows and dominates acid sulfate soils at the time of extraction (Figure 1). The growth of these weeds covers most of the soil surface because the land has not been used for rice cultivation for almost two years.

The determination of pyrite depth in acid sulfate soils was carried out by drilling at several depths. Subsequently, the drilled soil was spread over a flat dry surface and arranged according to the depth (Figure 2). The results of observations using the Munsell soil color chart showed changes in color at a depth of 0-10 cm colored 4/2

(a) (b)

Figure 1. (a) 'Purun tikus' (*Eleocharis dulcis*) and (b) 'papisangan' (*Ludwigia erecta*) growing at the research site

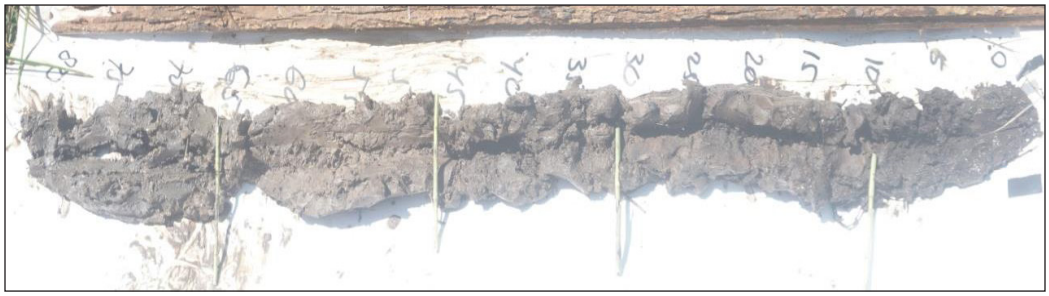


Figure 2. Acid sulfate soil depth profile 0-80 cm below the soil surface

10 yellow-red (YR) (dark grayish brown), 10-31 cm colored 4/2 5 YR (dark reddish gray), 31-46 cm colored 6/4 5 YR (light reddish brown), 46-65 cm colored 7/2 5 YR (pinkish gray), and >65 cm colored 6/3 7.5 YR (light brown).

The groundwater level in the land was found at a depth of 5 cm below the surface. Furthermore, the determination of pyrite using the rapid oxidation method in the field with 30% hydrogen peroxide (H_2O_2) solution was carried out, and the result showed that it was found at a depth of 63 cm from the ground surface.

Composting of Oyster Mushroom Baglog Waste

In the first phase of manufacturing oyster mushrooms, baglog waste obtained a total of 200 kg. Afterward, the waste was kept in the composting box with a length and width of 1.2 m long and a height of 0.8 m. The additional inputs were 10 kg of cow dung, chicken manure, and guano, as well as 3 kg of bran, which were stirred until evenly mixed.

The next phase was the addition of 300 mL of M-21 decomposer and molasses each into a 10 L bucket. Water was subsequently added until it reached 9 L, and the mixture of M-21, water, and molasses was stirred until evenly mixed and kept in the watering can. Furthermore, the mixture was poured over the composted material in the composting box, and the ingredients were stirred using a shovel until evenly mixed and covered with a burlap sack. On the next day, the temperature of the materials in the composting box was measured using a thermometer. Then, each material in the box was stirred evenly and covered again with a burlap sack.

The temperature measurement and mixing of materials in each composting box were carried out until the 21st day when the compost had matured. The maturity indicators of the composted material are: (1) there was a change in the original smell, as the compost emitted an odor like molasses (or no longer smells), (2) there was a change in the original color with blackish brown, and (3) the temperature was in the range of 28–35°C. Subsequently, the compost was kept in an airtight bag and stored for application in the field.

Fertilization

The compost was applied for two weeks based on the research treatment on prepared

land before planting. The additional fertilizers were mineral fertilizers such as urea (Pupuk Indonesia, Indonesia) for N fertilizer with a dose of 100 kg ha^{-1}, super phosphate ($Ca(H_2PO_4)$, namely 'SP-36', Petrokimia Gresik, Indonesia) for P fertilizer 25 kg ha^{-1}, and potassium chloride (KCl, Petrokimia Gresik, Indonesia) for K fertilizer 25 kg ha^{-1} (50% of the recommendation for rice fertilization). The urea fertilizer was given two times, 50 kg ha^{-1} each, during the planting and when the rice was four weeks after planting (WAP). Meanwhile, the SP-36 and KCl fertilizers were applied literally at the planting time. Recommendations for N, P, and K fertilization were sourced from the Ministry of Agriculture Number 40 of 2007 in West Martapura District (Ministry of Agriculture Republic Indonesia [MoA], 2007).

Seeds Preparation

The preparation of rice seeds (Inpara 10) was based on Permatasari et al. (2018). Ten (10) Inpara seeds were soaked in a salt solution prepared by incrementally adding salt to the water containing chicken eggs until they floated. The seeds that floated under this condition were discarded because they indicated an open grain, while the sinking ones were selected as they indicated to be full grain.

Chemical Properties of Acid Sulfate Soil and Compost of Oyster Mushroom Baglog Waste

The chemicals in the acid sulfate soils include pH, redox potential (Eh), organic C, N-mineral (NH_4^+, NO_3^-), available P, exchangeable K, Soluble Al, and Fe. In contrast, the chemical properties of the assessed compost and the reference for testing methods include pH, organic C, total N, C/N ratio, total P, total K, total Al, and Fe. The determination of selected soil chemical characteristics was conducted in moist conditions for approaching the field requirements. The methods are presented in Table 1.

There were various pH values in each sampled depth, but they were all very acidic. For example, organic C content at a depth of 0–10 cm and 10–31 cm of acid sulfate soils was classified as moderate, while at a depth of 31–45 cm, 45–65 cm, and >65 cm, it was classified as low (Eviati & Sulaeman, 2009). The chemical properties are presented in Table 2.

The content of NH_4^+ and NO_3^- at all sampled depths was classified as very low, while available P at 0–10 cm depths was classified as moderate, and available P at other soil sampled depths was classified as very low. The concentration of soluble Al and Fe at all sampled depths was very high, which is a characteristic of acid sulfate land. These criteria for classifying soil characteristics in this study were based on Eviati and Sulaeman's (2009) requirements for assessing soil properties.

The content of organic C, total N, C/N Ratio, P, K, Ca, Mg, Al, and Fe compost is in accordance with Indonesian National Standard [Standar Nasional Indonesia (SNI)] No.19-7030-2004. However, the results of pH measurement on the compost

did not meet SNI because the pH value was higher than the maximum according to the standard value. The results are presented in Table 3.

Although the pH did not meet the quality, the compost fulfilled the condition as being mature. It is supported by Meena et al. (2021), which stated that organic acids would neutralize their acid during the composting process, and the compost will mature with a pH usually between 6–8. In addition, a high pH has advantages in improving acidity, especially in South Kalimantan, which has acid sulfate soils and high acidity, where high compost pH can increase the pH of the soil. According

Table 1
Methods of chemical analysis of oyster mushroom baglog waste compost and acid sulfate soil

Chemical property	Method	Sample	Reference
pH (H_2O)	pH Electrode	Soil, compost	Neves et al. (2021)
Organic C	Walkley and Black	Soil, compost	Shamshuddin et al. (1994)
Total N	Micro-Kjeldahl	Soil, compost, plant tissue	Miller and Horneck (1997)
Total P	Ascorbic acid	Soil, compost, plant tissue	Raun et al. (1987)
Total K	Flame Photometry	Soil, compost, plant tissue	Juo (1978)
Al	Colorimetric (aluminon plus ascorbic acid)	Soil, compost	Abreu Jr. et al. (2003)
Fe	Ammonium acetate extracts	Soil, compost	Ure et al. (1993)
Eh	Eh electrodes	Soil	Rabenhorst et al. (2009)
N-mineral (NH_4^+, NO_3^-)	Morgan-Wolf Extract	Soil	Eviati and Sulaeman (2009)
Available P	Bray-I	Soil	Gutiérrez Boem et al. (2011)
Exchangeable K	Percolation	Soil	Matthews and Smith (1957)

Table 2
Chemical properties of acid sulfate soil at various depths (cm)

Chemical property	Unit	The amount by depths (cm)				
		0-10	10-31	31-45	45-65	>65
pH (H_2O)		4.75	4.67	4.61	4.60	4.06
Eh	mV	147.50	149.10	153.20	153.70	186.90
Organic C	mg kg^{-1}	3.09	2.89	1.64	1.44	1.32
NH_4^+	mg kg^{-1}	1.30	1.21	1.72	0.94	0.41
NO_3^-	mg kg^{-1}	1.43	1.40	1.43	1.38	1.28
Available P	mg kg^{-1}	9.30	4.95	2.19	2.26	2.16
Exchangeable K	cmol (+) kg^{-1}	0.16	0.15	0.16	0.14	0.16
Soluble Al	mg kg^{-1}	272.24	339.33	300.09	317.90	381.56
Soluble Fe	mg kg^{-1}	306.68	339.92	364.79	341.25	432.57

Table 3
Chemical properties of oyster mushroom baglog waste compost

Chemical property	Unit	Amount
pH (H_2O)		9.80
Organic C	mg kg^{-1}	21.95
Total N	mg kg^{-1}	1.10
C/N ratio		19.96
P_2O_5	mg kg^{-1}	1.99
Potassium oxide (K_2O)	mg kg^{-1}	0.35
Ca	mg kg^{-1}	4.44
Mg	mg kg^{-1}	0.30
Al	mg kg^{-1}	0.0017
Fe	mg kg^{-1}	0.0038

to Saputra and Sari (2021), applying an ameliorant with a pH of 8.4 can increase the pH of peat and tidal swamp soils because they contain Ca and Mg. These elements will replace the H^+ position on the colloidal surface to neutralize acidity.

Planting

The rice seedlings were transplanted to experimental plots after 12 days in the nursery with as much as one seed per planting hole (single planting), shallow, and the root position forming the letter L. Planting was carried out with a spacing of 30 x 30 cm and the water treatment in the rice fields was in a saturated condition with water layer of 3 cm above the soil surface (*macak-macak*).

Observation

Observations were made five times, at the early stage of 0 weeks after planting (0 WAP) or before applying urea, SP-36, or KCl fertilizer, full vegetative phase (8 WAP), early panicle emergence (9 WAP), filling panicles (12 WAP), and harvest phase (15 WAP). Also, soil sampling was conducted in every growth stage with purposive sampling. Each experimental unit obtained 250 g and was analyzed in the laboratory. Determination of the ammonium (NH_4^+) and nitrate (NO_3^-) levels using a Morgan Wolf extract was measured with a wavelength of 636 nm and 494 nm (Eviati & Sulaeman, 2009). Meanwhile, the determination of available P utilized Bray-I methods (Gutiérrez Boem et al., 2011) and the exchangeable K content used percolation (Matthews & Smith, 1957).

The observation of nutrient uptake was carried out at the harvest phase (15 WAP). The Kjeldahl method was only applicable to plant tissue to digest N contents (Miller & Horneck, 1997). Digestion in plant tissue of P contents was carried out using mixture of nitric acid and perchloric acid (HNO_3-$HClO_4$) digest and orthophosphate as phosphorus (PO_4-P) in a dilute acid extract (DAEP), and the contents of P in digested solution were determined using the

ascorbic acid method (Raun et al., 1987). K contents were divided using HNO_3-$HClO_4$ acid digest, and the contents of K in digested solution were determined using the flame photometry method (Juo, 1978).

Sampling Data Processing

Variance analysis was carried out on the observed variables using the GenStat (12th edition) application to examine the effect of the application of oyster mushroom baglog waste on changes in soil N, P, K nutrient availability and uptake. Prior to this analysis, the homogeneity of variance was tested. When the analysis showed that the compost application significantly affected the observed variables (P<0.05), a different treatment test was subsequently carried out. The different treatment tests need to be taken using Duncan's Multiple Range Test (DMRT) at a level of 5% (Duncan, 1955).

RESULTS AND DISCUSSION

The Dynamics of NH_4^+ and NO_3^-

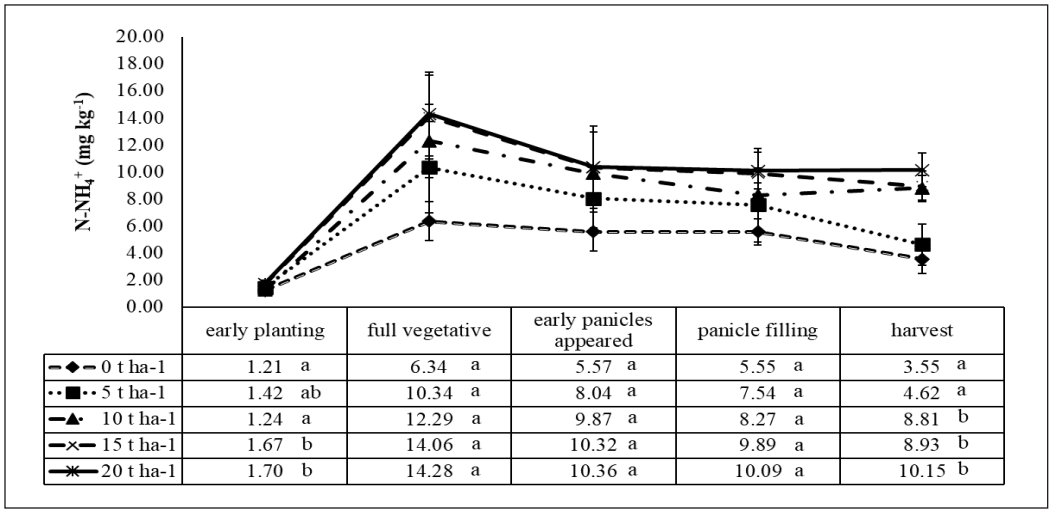
The observations made at five different growth stages of early planting, full vegetative, early panicle emergence, panicle filling, and harvesting showed variation in the availability of NH_4^+ and NO_3^- in acid sulfate soils. The results for the amount of NH_4^+ and NO_3^- are presented in Figures 3a and 3b.

The compost with a dose of 20 t ha^{-1} could improve the highest N-mineral (NH_4^+ and NO_3^-) contents in acid sulfate soils. This result is in line with Jumar et al. (2021), which stated that utilization of *Pleurotus ostreatus* substrates compost with a dose of 20 t ha^{-1} improved the chemical properties in terms of N-mineral in acid sulfate soils. Furthermore, Burhan and Proyogo (2019) claimed that the greater dose of baglog applied to the planting media enhanced the fertility and developed better plant growth of *Allium ascalonicum* L. The compost of baglog waste dose that significantly raised the plant growth ranged from 10-30 t ha^{-1} (Prabowo et al., 2020; Saputra & Sari, 2021). Moreover, Bonanomi et al. (2020) showed that organic amelioration enhanced fertility and improved a beneficial soil microbiota equipped for supporting high plant yield under an intensive agricultural system.

The availability of the minerals (NH_4^+ than NO_3^-) is due to the stimulating effect of N fertilizer (urea) and organic matter application under the rice intensification technique. The greater availability of NH_4^+ than NO_3^- was due to the chemical reactions catalyzed by enzymatic activity, which occurs after the fertilizer contact with the soil. Nitrogen in soil is converted to NH_3 and shortly after that to NH_4^+ (Marchezan et al., 2020). The process of transforming urea into NH_4^+ was influenced by the soil organic matter, as its high content quickens the transformation process. Therefore, the improvement of the matter could enhance the ammonification process that changes organic N to NH_4^+ (Saidy, 2018). This study also supported the compost of baglog oyster mushroom waste at a dose of 20 t ha^{-1}, which had the highest dose with more NH_4^+ content of 14.28 mg kg^{-1}. Therefore, the formation

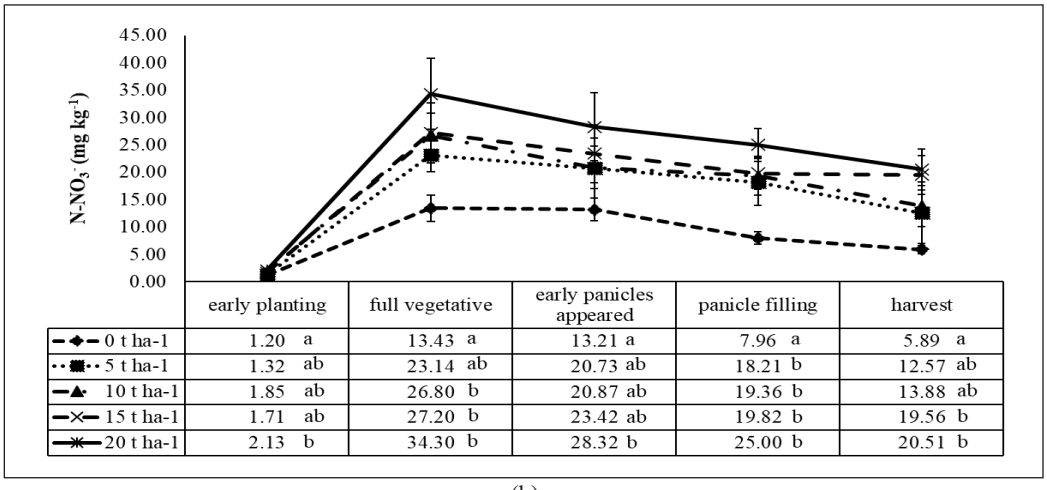
speed of the minerals was certainly good as the soil could meet the N needs of rice plants.

The levels of NH_4^+ were lower than NO_3^- due to the absorption of N by rice plants and nitrification (transformation of NH_4^+ to NO_3^-) processes. According to Figure 3, the NO_3^- the amount in the plants was higher than NH_4^+ because the rice cultivation in this study used an intensification technique, causing the paddy soils to be more aerobic. The nitrification process cannot be separated from the aerobic condition of the soil, which allows



	early planting	full vegetative	early panicles appeared	panicle filling	harvest
0 t ha-1	1.21 a	6.34 a	5.57 a	5.55 a	3.55 a
5 t ha-1	1.42 ab	10.34 a	8.04 a	7.54 a	4.62 a
10 t ha-1	1.24 a	12.29 a	9.87 a	8.27 a	8.81 b
15 t ha-1	1.67 b	14.06 a	10.32 a	9.89 a	8.93 b
20 t ha-1	1.70 b	14.28 a	10.36 a	10.09 a	10.15 b

(a)



	early planting	full vegetative	early panicles appeared	panicle filling	harvest
0 t ha-1	1.20 a	13.43 a	13.21 a	7.96 a	5.89 a
5 t ha-1	1.32 ab	23.14 ab	20.73 ab	18.21 b	12.57 ab
10 t ha-1	1.85 ab	26.80 b	20.87 ab	19.36 b	13.88 ab
15 t ha-1	1.71 ab	27.20 b	23.42 ab	19.82 b	19.56 b
20 t ha-1	2.13 b	34.30 b	28.32 b	25.00 b	20.51 b

(b)

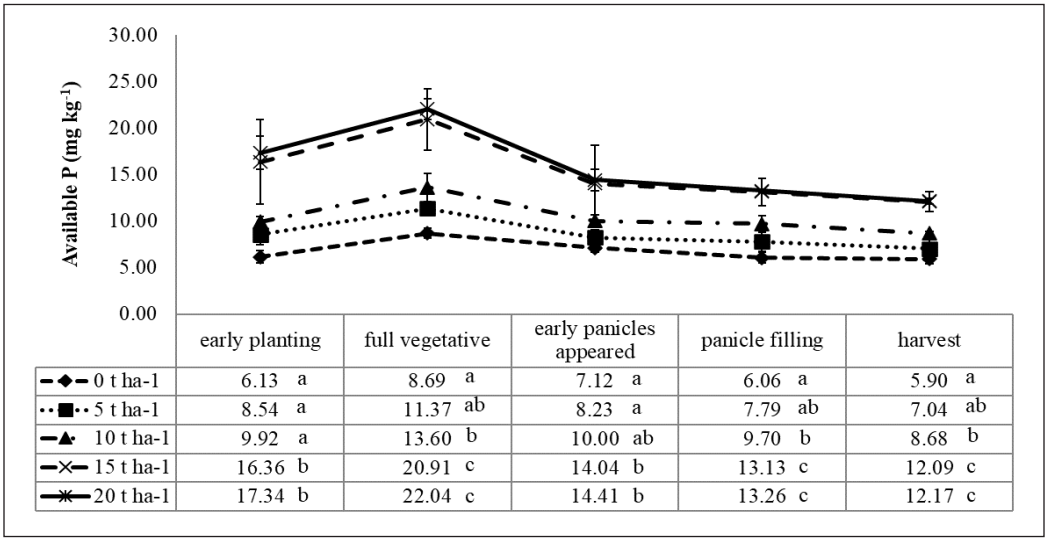
Figure 3. The dynamics of (a) NH_4^+ and (b) NO_3^- in several rice stadia cultivated under rice intensification and ameliorated by the compost of oyster mushroom baglog waste

Note. The line above the diagram is the standard error of the treatment (n = 5). Numbers followed by the same letter at the same rice stadia indicate that the treatment has a no different effect based on Duncan's Multiple Range Test (DMRT) at the level of 5%

nitrifying bacteria to function properly and increase the amount of NO_3^- than NH_4^+ (Khotimah et al., 2020; Sugiarta, 2016). The alternate wetting and drying treatment on the rice plants (as an intensification technique) improved soil NO_3^- content, nitrification processes, N absorption, and accumulation (Chunmei et al., 2020). Improving soil oxygen (O_2) (more aerobic) conditions aid the conversion of soil N cycling and contributes to enhancing the N absorption and accumulation by rice plants in paddy fields. The key to the intensification technique is intermittent periodical irrigation of keeping shallow water depths (*macak-macak*) and intense application of composted organic amendment (Arif et al., 2019). This technique aims to alter the condition of paddy soils to be more aerobic and control the nitrification balance.

The Dynamics of Available P

The observations made at the five different growth stages of early planting, full vegetative, early panicle emergence, panicle filling, and harvesting showed fluctuations of available P in acid sulfate soils. The results are presented in Figure 4. The compost with a dose of 20 t ha^{-1} could improve the highest available P. This result is in line with Jumar et al. (2021), which stated that the use of *Pleurotus ostreatus* substrates compost with a dose of 20 t ha^{-1} improved the chemical properties in term of available P in acid sulfate soils. Phuong et al. (2020b) also claimed that compost in these soils successfully improved available P by magnifying labile P contents with 100% and 200% doses of 10 g kg^{-1} and 20 g kg^{-1}. In addition, the compost of baglog waste mixed with wood biochar could increase



	early planting	full vegetative	early panicles appeared	panicle filling	harvest
0 t ha-1	6.13 a	8.69 a	7.12 a	6.06 a	5.90 a
5 t ha-1	8.54 a	11.37 ab	8.23 a	7.79 ab	7.04 ab
10 t ha-1	9.92 a	13.60 b	10.00 ab	9.70 b	8.68 b
15 t ha-1	16.36 b	20.91 c	14.04 b	13.13 c	12.09 c
20 t ha-1	17.34 b	22.04 c	14.41 b	13.26 c	12.17 c

Figure 4. The dynamics of available P in several rice stadia using rice intensification technique given compost of oyster mushroom baglog waste

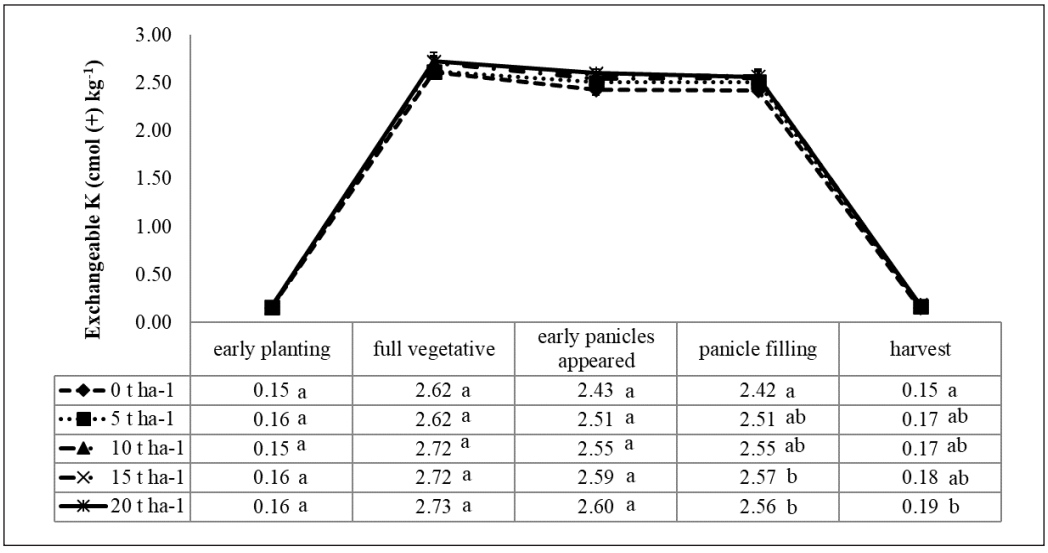
Note. The line above the diagram is the standard error of the treatment (n = 5). Numbers followed by the same letter at the same rice stadia indicate that the treatment has a no different effect based on Duncan's Multiple Range Test (DMRT) at the level of 5%

the photosynthetic rate and plant growth performance (Seehausen et al., 2017).

The increase of available P is due to the addition of the compost combination of oyster mushroom baglog waste and SP-36 fertilizer. The combination of phosphorus pentoxide (P_2O_5) from SP-36 fertilizer directly increased available P, but the main problem in acid sulfate soils is the high solubility of Fe and Al (Jumar et al., 2021). In accordance with the data in Table 2, the content of dissolved Fe and Al was categorized as very high. Therefore, the organic matter from the compost was very important in order not to disturb the available P in the soil. Furthermore, according to Eusterhues et al. (2005), the application of organic matter, such as compost, can reduce Fe^{3+} to Fe^{2+}, which is highly reactive to organic matter. Therefore, the material given in the form of compost could cover the toxicity of Fe, which binds important nutrients to the soil. Likewise, soluble Al could be neutralized by adding organic matter to the soil due to the binding of Al^{3+} by organic acid functional groups, such as humic acid. Therefore, the plants demanded available P to accelerate the flowering process and panicle filling. Moreover, Zhang et al. (2012) confirmed that this chemical is important because it accelerates the maturity of panicle filling, thereby improving the quality of the rice.

The Dynamics of Exchangeable K

The variations in the availability of exchangeable K in acid sulfate soils are shown in Figure 5. The compost with a dose of 20 t ha^{-1} showed that the exchangeable K content was effectively available. Hanifa



	early planting	full vegetative	early panicles appeared	panicle filling	harvest
0 t ha-1	0.15 a	2.62 a	2.43 a	2.42 a	0.15 a
5 t ha-1	0.16 a	2.62 a	2.51 a	2.51 ab	0.17 ab
10 t ha-1	0.15 a	2.72 a	2.55 a	2.55 ab	0.17 ab
15 t ha-1	0.16 a	2.72 a	2.59 a	2.57 b	0.18 ab
20 t ha-1	0.16 a	2.73 a	2.60 a	2.56 b	0.19 b

Figure 5. The dynamics of exchangeable K in several rice stadia using rice intensification technique given compost of oyster mushroom baglog waste

Note. The line above the diagram is the standard error of the treatment (n = 5). Numbers followed by the same letter at the same rice stadia indicate that the treatment has a no different effect based on Duncan's Multiple Range Test (DMRT) at the level of 5%

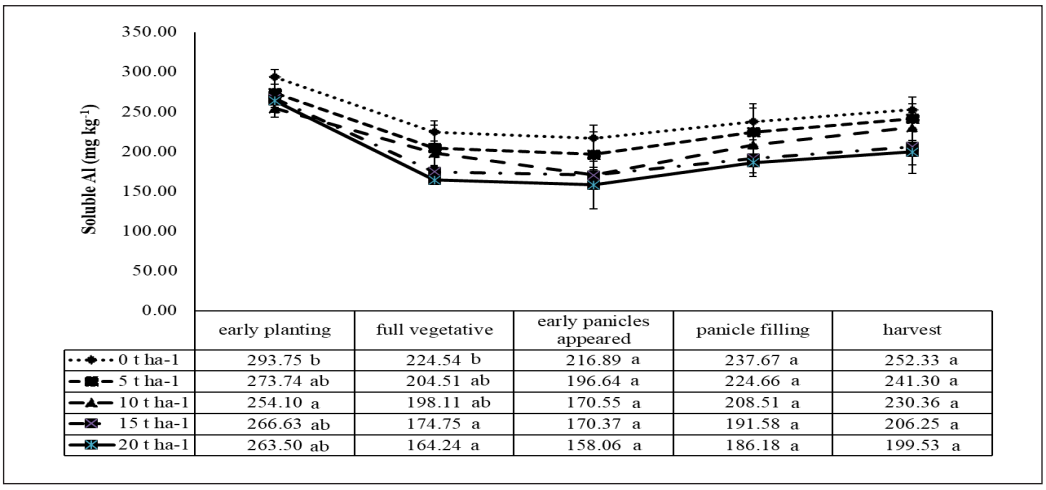
et al. (2019) stated that plants consistently consumed N-mineral and available P until they developed towards maturity, while exchangeable K contents are particularly required in the reproductive growth stages of corn plants. Phuong et al. (2020a) affirmed that the higher application dose of compost in acid sulfate soils could precisely magnify K^+ elements, bringing about higher exchangeable K than in unameliorated soils. Therefore, the compost amendment by 20% could give nutrients fitting to establish plant growth and meet the fertilizer prerequisites of commercial seedlings (Meng et al., 2019).

There was an increase in exchangeable K because of the compost combination of oyster mushroom baglog waste and KCl fertilizer. The treatment of 20 t ha^{-1} of the waste in this research succeeded increasing the exchangeable K in acid sulfate soils. The provision of KCl in the rice intensification system plays a very important role in increasing the exchangeable K. This is in accordance with Isnaini (2005), which stated there was a positive correlation in soil between total N and NH_4^+, where the content of the latter is more significant in the provision of exchangeable K. The increased of exchangeable K in maize plants by the application of organic amendments was strongly influenced by soil moisture mechanism (Manolikaki & Diamadopoulos, 2019; Rogovska et al., 2014). Organic amendments could improve the porosity of soils, which could generate the rearrangement of pore-size distribution and aggregation in soils, thereby contributing to higher soil water retention (Guo et al., 2020).

The Dynamics of Soluble Al and Fe

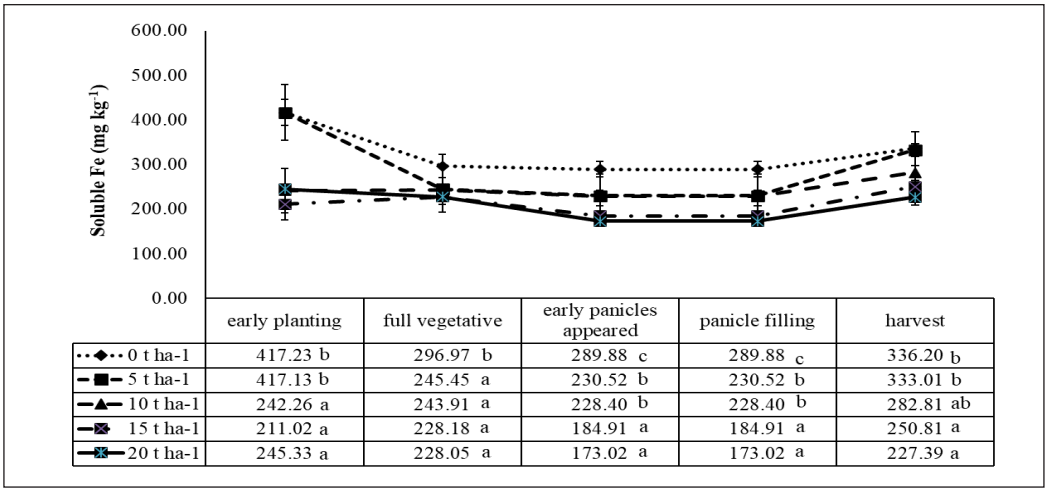
The observation results showed a fluctuation in Soluble Al and Fe levels at five different growth stages. The results are presented in Figures 6a and 6b. The compost as an organic matter plays an important role in managing the solubility of some metals. The more organic matter dissolved in soils, the less amount of soluble Al in plants because the dissolved organic matter (DOM) will buffer the free state of Al and Fe with DOM-metal bound (Stirling et al., 2020; Watmough & Orlovskaya, 2015). Zanin et al. (2019) further stated that organic matters are referred to as redox reactive, which can reduce metal ionic compounds, including Fe^{3+}. In addition, it has been verified that they can accelerate the reduction of Fe (III)-oxide in sediments and bioreduction of Fe (III) minerals in the soil in a dissolved and stable state.

The main source of dissolved organic carbon (DOC) in soil usually originated from root exudates. Rice growth promotes extra photosynthesized (newly-derived) C into soil C pools compared to unplanted land, reflecting the discharge of root exudates from rice roots (Ge et al., 2012). The previous studies stated that within higher soil N, the release of C from the roots is elevated since N uptake through rice flora is stronger in the prophase of the growth degree (Ge et al., 2015). Said-Pullicino et al. (2016) affirmed that the soil-derived DOC should stimulate the reductive dissolution of Fe (hydr)oxides by presenting electrons from organic matter degradation to Fe-lowering micro-organisms. Therefore, the



	early planting	full vegetative	early panicles appeared	panicle filling	harvest
0 t ha-1	293.75 b	224.54 b	216.89 a	237.67 a	252.33 a
5 t ha-1	273.74 ab	204.51 ab	196.64 a	224.66 a	241.30 a
10 t ha-1	254.10 a	198.11 ab	170.55 a	208.51 a	230.36 a
15 t ha-1	266.63 ab	174.75 a	170.37 a	191.58 a	206.25 a
20 t ha-1	263.50 ab	164.24 a	158.06 a	186.18 a	199.53 a

(a)



	early planting	full vegetative	early panicles appeared	panicle filling	harvest
0 t ha-1	417.23 b	296.97 b	289.88 c	289.88 c	336.20 b
5 t ha-1	417.13 b	245.45 a	230.52 b	230.52 b	333.01 b
10 t ha-1	242.26 a	243.91 a	228.40 b	228.40 b	282.81 ab
15 t ha-1	211.02 a	228.18 a	184.91 a	184.91 a	250.81 a
20 t ha-1	245.33 a	228.05 a	173.02 a	173.02 a	227.39 a

(b)

Figure 6. The dynamics of (a) soluble Al and (b) Fe in several rice stadia cultivated under rice intensification and ameliorated by the compost of oyster mushroom baglog waste
Note. The line above the diagram is the standard error of the treatment (n = 5). Numbers followed by the same letter at the same rice stadia indicate that the treatment has a no different effect based on Duncan's Multiple Range Test (DMRT) at 5%

application of the compost, which provides N uptake availability, increased the root exudates and consequently reduced soluble Fe and Al uptake by promoting more DOC in paddy soils.

The dynamic levels of P availability also influenced the availability of soluble Al and Fe in rice plants. The regular concept stated that the reduction of Fe (III) within the soil could solubilize P, likely through desorption approaches mediated by using subsequent biological assimilation, precipitation, and resorption by Fe (III) species (Peretyazhko & Sposito, 2005). Powerful decreasing

conditions with the regular augmentation of organic matter amelioration are required to increase P solubility in these Fe-rich tropical soils (Lin et al., 2018). Also, Khan et al. (2019) showed microbial-mediated Fe (III) reduction was intensified through labile organic C compounds, which acted as energy resources and electron donors. The discharge of available P through Fe reduction followed the way of Fe (III) peaks, DOC, or pH, and was observed through a decrease in iron-bound P (Fe-P). It indicated that Fe-P is the main supply of P in the paddy soils. Therefore, the application of oyster mushroom baglog waste compost in this study, which provides the P availability, reduced the soluble Al and Fe levels in paddy fields as the level of available P improved.

Relationship Between Nutrient Availability and Nutrient Absorbed by Rice Plants

The relationship between nutrient availability at the harvest phase and rice plant uptake was determined using a

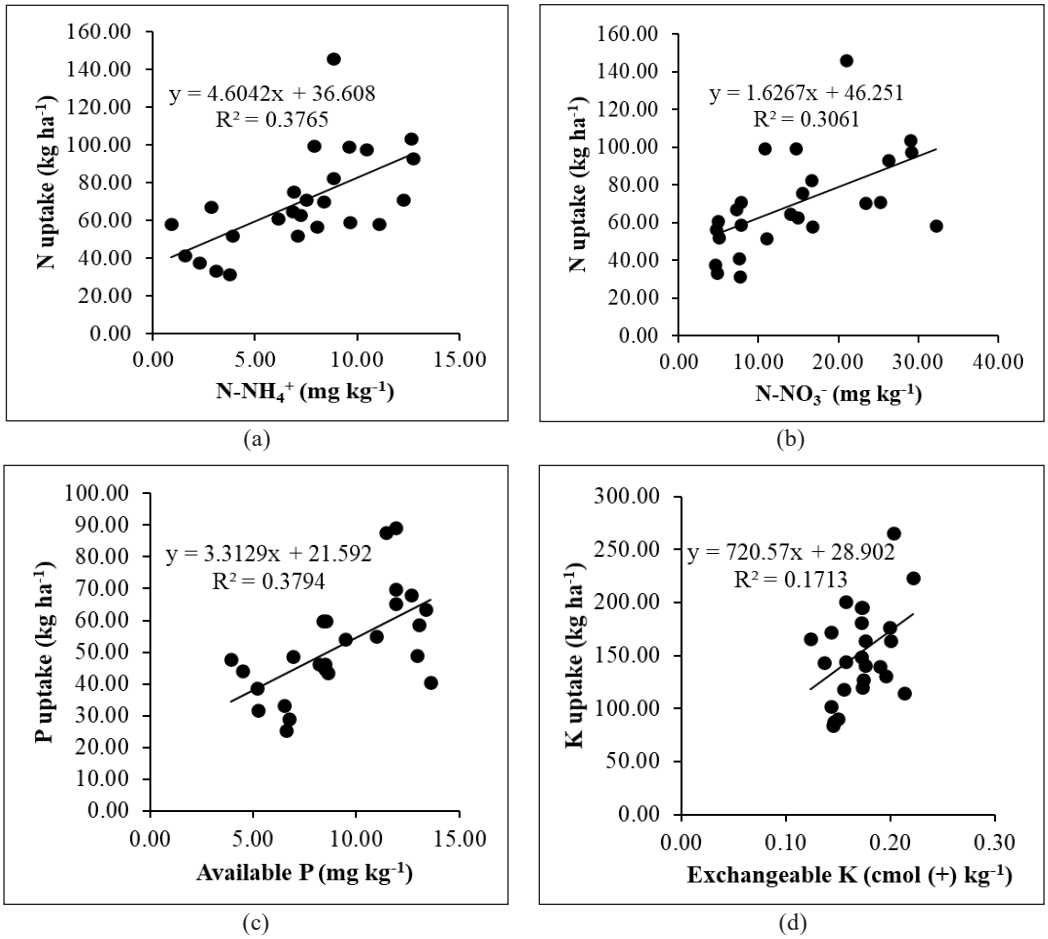


Figure 7. Correlation between: (a) NH_4^+ and N uptake; (b) NO_3^- and N uptake; (c) Available P and P uptake, and (d) exchangeable K and K uptake of rice

correlation test. The uptake illustrates the number of nutrients absorbed by plants, obtained by multiplying the dry weight of nutrient concentrations in the plant tissue. Figure 6 shows the correlation between the availability of N, P, and K and their nutrient uptake in rice plants.

There was a positive correlation between the availability of NH_4^+ in acid sulfate soils at the harvest stage and the N uptake using the intensification technique with a correlation coefficient value (*r*) of 0.6136 (Figure 7a). Likewise, the availability of NO_3^- at the harvest stage and the N uptake with a coefficient value (*r*) of 0.5533 (Figure 7b) indicated a moderate level of close relationship (Schober et al., 2018). Furthermore, a moderate level of a close relationship with a positive correlation coefficient value (*r*) of 0.6160 was found in the available P with the uptake (Figure 7c). Moreover, the exchangeable K with the K uptake of rice had a coefficient value (*r*) of 0.4139 (Figure 7d) which indicated the criteria of moderate relationship closeness (Schober et al., 2018).

The higher the NH_4^+, NO_3^-, available P, and exchangeable K, the more N, P, and K uptake using the intensification technique. The compost provision of oyster mushroom baglog waste provided N, P, and K in available forms. Hence, the roots can optimally absorb these nutrients. It is supported by the data presented in Figures 3 to 5 that the dose of compost given increased with the availability of N (NH_4^+ and NO_3^-), P, and K at all stages using the intensification technique. Furthermore, Sopha et al. (2015) stated that tissue analysis results increased with the growth and production of plants.

CONCLUSION

The compost supplied N, P, and K in available forms and reduced the amount of soluble Al and Fe; hence, the roots can absorb these nutrients optimally. Also, the compost increased the availability of essential macro-nutrients and uptake in plants under the rice intensification technique from early planting stages to the harvest phase.

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Short Communication

Feed Intake and Apparent Nutrient Digestibility of Growing Rabbits Fed *Asystasia gangetica* with Different Levels of Corn

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ABSTRACT

This study investigated the effect of different corn levels on intake and digestibility in rabbits. The rabbits were divided into four groups and given *Asystasia gangetica ad libitum* as a basal diet and supplemented with either 80 g (T1), 60 g (T2), 40 g (T3), or 0 g (T4) corn/head/day. Rabbits fed with the T4 diet demonstrated a higher intake/kg metabolic weight than the other treatments. Nevertheless, rabbits fed with T4 and T1 diets depicted the lowest and highest digestibility, respectively. In conclusion, apparent nutrient digestibility was significantly improved in growing rabbits following supplemental feeding with a diet containing 60-80 g of corn.

Keywords: *Asystasia gangetica*, corn, feed intake, nutrient digestibility

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INTRODUCTION

Asystasia gangetica is an important perennial herb and weed widely distributed in tropical Asia and Africa. It is widely grown as ground cover in Malaysian and Indonesian oil palm plantations due to its tolerance to low soil fertility and shade (Ramdani et al., 2017). Although *A. gangetica* is considered a serious environmental and agricultural weed, it has high nutritional

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value as an animal feed and contains several biologically active substances with various medicinal properties (Ali et al., 2021). For instance, the leaves of *A. gangetica* contain 19.3% crude protein (CP), which is higher than the protein requirements for ruminants and pseudo-ruminants (Khalil, 2016). Given the high protein value, *A. gangetica* is already utilised as forage for ruminants in South-East Asia either by grazing or cut and then used in stall-feeding (Ali et al., 2021).

According to Amata and Okorodudu (2016), a ratio of 50:50 mixture of *Centrocema pubescens* and maize concentrate is necessary for maximum performance. In another study, Rahman et al. (2020a) observed that a rabbit's diet, which includes half concentrate and half-dwarf Napier grass, resulted in the best growth and performance. Nonetheless, the effect of *A. gangetica* with corn supplementation on rabbits remains underreported and unclear. Therefore, this study was conducted to investigate the effects of different dietary levels of corn on intake and digestibility in rabbits fed with *A. gangetica* as a basal diet.

MATERIALS AND METHODS

Study Site and Experimental Design

The experiment was conducted at Agro Techno Park, Universiti Malaysia Kelantan (UMK), Jeli Campus, Kelantan; according to the procedures approved by the UMK Animal Care and Use Committee (UMK/FIAT/ACUE/FYP/1/2020). A total of 16 weaned rabbits with an approximate age of two months of both sexes were bought from a local supplier. The mean (± standard deviation) body weight of the rabbits was 1387.5 (± 145.5 g). The rabbits were adapted to the new environment for seven days of pre-data collection before experimenting. During the acclimatisation period, the rabbits were fed commercial rabbit pellets. The rabbits were divided into four groups and given *Asystasia gangetica ad libitum* as a basal diet and supplemented with either 80 g (T1), 60 g (T2), 40 g (T3), or 0 g (T4) corn/head/day. *A. gangetica* was offered to rbbits with a target amount of 10% refusal. The rabbits consumed about 80.0 g of corn as a sole source of feed prior to the experiment. Hence, rabbits in the control group (T1) were offered corn at 80 g/d/rabbit with the inclusion of *A. gangetica*.

A digestibility trial with rabbits was conducted for 28 days: 21 days as the preliminary period and seven days for the collection period. In the preliminary period, the same amount of test feed administered during the collection period was provided daily. *Asystasia gangetica* and corn were offered twice daily (9:00 a.m. and 5:00 p.m.) after dividing into two equal portions. The corn was bought from a local supplier, while naturally grown *A. gangetica* was collected daily at the vegetative growth stage from Agro Techno Park, UMK. The corn and *A. gangetica* were offered in separate feeders. The samples of *A. gangetica* were obtained daily to determine dry matter (DM) intakes since there would be variation in the DM daily. The rabbits were housed individually in metal stacked cages (size of the cage: 45 cm height × 40 cm wide × 50 cm length). Each cage was allocated a feed trough

and automatic water nipples. During the collection period, plastic gauze was placed under each cage for faeces collection.

Parameters Measured and Chemical Analysis

The feed offered and refusals were recorded to estimate intake, and the samples were collected daily to determine dry weight. Before the morning feed, rabbits were weighed at the beginning of the experiment, at 1-week intervals, and the end. The total weight gain of a rabbit was calculated by subtracting the initial weight from its final weight. During the collection period, offered feeds, refusals and faeces were recorded, and representative samples were stored in a freezer. Prior to chemical analysis, each rabbit's faeces samples were thawed and mixed to obtain a representative sample. After that, the representative samples of faeces and feeds were dried and analysed for dry matter (DM), ash, CP, ether extract (EE), and crude fibre (CF) following the method of the Association of Official Analytical Chemists (AOAC) (2000). All data were analysed using a one-way analysis of variance in Statistical Product and Service Solutions (SPSS) software (version 23). Mean comparisons were conducted using the Duncan Multiple Range Test (DMRT) at $p < 0.05$.

RESULTS AND DISCUSSION

Nutritive Value

The chemical composition of *A. gangetica* and corn is presented in Table 1. The DM (16.6%) and EE (4.3%) contents of *A. gangetica* in this study were higher compared to the estimates reported by Rahman et al. (2020b). These differences might be due to several factors, including botanical fractions and plant maturity (Koca & Erakul, 2016). Notably, the EE contents of both ingredients used in this study were higher than the EE requirements (2.5-4.0%) for adult rabbits, as reported by the National Research Council (NRC) (1977). In contrast, the organic matter (OM) content of *A. gangetica* is similar to the findings by Rahman et al. (2020b). The CP content is lower compared to the reports by Khalil (2016), who found that the CP content in *A. gangetica* was 23.2%.

Table 1
Chemical composition (%, DM) of Asystasia gangetica *and corn*

Nutrients	*Asystasia gangetica*	Corn
Dry matter	16.6	90.0
Organic matter	91.8	93.4
Crude protein	18.5	8.0
Ether extract	4.3	7.8
Crude fibre	21.1	0.5
Ash	8.2	6.6
Metabolisable energy (MJ/kg DM)$^{\beta}$	9.1	12.9

Note. $^{\beta}$data obtained from the secondary data (Sudin et al., 2005); MJ = Megajoule; DM = Dry matter

Cheeke (1987) reported that rabbits require 14% CF in their diet to maintain gut motility. The *A. gangetica* in this study contained 21.1% CF, which is lower than the findings of Khalil (2016). Meanwhile, the ash content is consistent with Sudin et al. (2005), who reported that *A. gangetica* contained 10.9% and 6.7% ash content in their young shoot and mature plants, respectively.

Nutrient Intake

Rabbits fed with the T4 diet demonstrated higher ($p < 0.05$) DM intake/kg metabolic weight (MW, equal to $W^{0.75}$) (63.0 g), followed by those fed with T1 (50.4 g), T3 (46.5 g), and T2 (45.9 g) diets (Table 2). The decrease in daily DM intake/kg MW was more significant when the level of CF in diets was low. Similarly, the OM intake/kg MW by rabbits fed diets T1, T2, and T3 were similar to the OM intake by these three groups, which was significantly ($p < 0.05$) lower compared to those fed the T4 diet. Meanwhile, rabbits fed with the T4 diet depicted significantly higher ($p < 0.05$) CP and CF intakes, followed by rabbits fed with T3, T2, and T1 diets. Fibre can be efficiently utilised in rabbits' diets since it is vital for preventing gut problems, diarrhoea, and mortality in animals (Ikyume et al., 2019). In contrast, rabbits fed with the T4 diet consumed 173.4 kcal metabolisable energy (ME) per day, which accounted for 89% of

Table 2
Dry matter and nutrient intakes by rabbits fed with Asystasia gangetica *with different corn levels*

Parameter	Treatment (mean ± standard deviation)				*p*-value
	T1	T2	T3	T4	
Daily intake (g)					
Asystasia gangetica	$21.7^{d} \pm 2.48$	$25.3^{c} \pm 3.29$	$42.8^{b} \pm 3.44$	$79.5^{a} \pm 3.39$	0.000
Corn	$47.7^{a} \pm 7.37$	$32.9^{b} \pm 3.01$	$20.0^{c} \pm 0.00$	$0.0^{d} \pm 0.00$	0.000
Total dry matter	$69.4^{b} \pm 6.26$	$59.1^{c} \pm 3.18$	$61.8^{c} \pm 2.23$	$79.5^{a} \pm 4.51$	0.000
Total dry matter/kg $W^{0.75}$	$50.4^{b} \pm 7.28$	$45.9^{b} \pm 3.33$	$46.5^{b} \pm 3.36$	$63.0^{a} \pm 5.50$	0.002
Total organic matter	55.1 ± 18.77	54.8 ± 2.94	56.4 ± 3.06	73.0 ± 4.14	0.062
Total organic matter/kg $W^{0.75}$	$39.0^{b} \pm 11.06$	$42.6^{b} \pm 3.06$	$42.4^{b} \pm 3.96$	$57.8^{a} \pm 5.04$	0.007
Total crude protein	$7.8^{c} \pm 0.66$	$7.4^{c} + 0.58$	$9.4^{b} \pm 0.61$	$14.7^{a} \pm 0.83$	0.000
Total crude protein/kg $W^{0.75}$	$5.7^{c} \pm 0.91$	$5.8^{c} \pm 0.62$	$7.1^{b} \pm 0.73$	$11.6^{a} \pm 1.010$	0.000
Total ether extract	4.7 ± 0.47	3.7 ± 0.21	5.4 ± 4.34	3.4 ± 0.19	0.588
Total ether extract/kg $W^{0.75}$	3.4 ± 0.47	2.9 ± 0.18	4.1 ± 3.41	2.7 ± 0.23	0.681
Total crude fibre	$4.9^{c} \pm 0.57$	$5.5^{c} \pm 0.80$	$9.1^{b} \pm 0.70$	$16.7^{a} \pm 0.95$	0.000
Total crude fibre/kg $W^{0.75}$	$3.5^{c} \pm 0.75$	$4.3^{c} \pm 0.76$	$6.9^{b} \pm 0.79$	$13.3^{a} \pm 1.16$	0.000
ME from *Asystasia gangetica* (kcal)¶	$47.2^{c} \pm 5.39$	$55.1^{c} \pm 7.16$	$93.2^{b} \pm 7.48$	$173.4^{a} \pm 7.36$	0.000
ME from corn (kcal)¶	$147.0^{a} \pm 22.73$	$101.6^{b} \pm 9.29$	$61.7^{c} \pm 0.00$	-	0.000
Total ME (kcal)¶ intake	$194.3^{a} \pm 19.97$	$156.7^{c} \pm 8.14$	$154.9^{c} \pm 7.48$	$173.4^{b} \pm 7.37$	0.000

Note. $W^{0.75}$ = Metabolic weight; Means with different superscripts in a row differ significantly ($p < 0.05$); ¶Metabolisabe energy intake was calculated using energy values for the ingredients obtained from Sudin et al. (2005)

the ME intake in the T1 group ($p < 0.05$). Approximately 76%, 65%, 40%, and 0% of the total daily ME intake for T1, T2, T3, and T4 groups was contributed by corn, whereas *A. gangetica* contributed nearly 24%, 35%, 60%, and 100%, respectively. Higher DM and nutrient intakes were observed for rabbits fed with the T4 diet than in the other treatments, which aligns with the findings of Yu and Peter (1996), who reported that feed intake increased with increasing dietary fibre levels.

Nutrient Digestibility and Growth

The DM and OM digestibility were higher ($p < 0.05$) in rabbits fed with diets T1 and T2 compared to those fed diets T3 and T4. The DM digestibility observed in this study ranged from 80.2% to 89.2%, and these values are similar to the reports of Ikyume et al. (2019) but higher than the findings of Rahman et al. (2020b). Likewise, the OM digestibility values in this study ranged from 48.4% to 74.0%, aligning with previous findings (Rahman et al., 2020b). The CP digestibility of the rabbits was not significantly ($p > 0.05$) different among the dietary groups, ranging from 47.4% to 56.9%. These results are lower than the earlier outcomes of Ikyume et al. (2019), who had fed *Leucaena leucocephala* and *Panicum maximum* forages to weaned rabbits.

The EE digestibility was significantly ($p < 0.05$) higher in rabbits fed with diets T1 and T2 than those fed diet T4, whereas those fed diets T3 and T4 were statistically similar. A decreasing trend was observed in EE digestibility with a reducing rate of corn in the diet, except for diet T1. The EE digestibility values for diets T1 and T2 are consistent with the reports by Ikyume et al. (2019), but they were lower for diets T3 and T4. The CF digestibility was higher ($p < 0.05$) in rabbits fed with the T1 diet compared with those fed diets T3 and T4, whereas no statistical difference was observed between those fed diets T2, T3, and T4. This finding might be attributed to the difference in the physical forms of the experimental diets, thereby contributing to the variation in the degree of digestibility of the cell wall. In other words, diet T1 is significantly easier to digest, probably due to its higher corn contents, which contain less fibre (0.5%) compared to *A. gangetica* (21.1%) (Table 1).

Rabbits fed with diet T1 showed the highest value of final body weight (BW) (1617.5 g), while those fed diet T4 presented the lowest (1412.5 g). Rabbits fed with diet T1 showed significantly ($p < 0.05$) higher BW gain than those fed diet T4. Meanwhile, rabbits fed with diet T4 did not reveal any significant growth throughout the four weeks despite consuming 79.5 g of *A. gangetica* DM daily, resulting in a total protein consumption of 14.7 g and ME 173.4 kcal/day. It might be explained by the high content of structural carbohydrates such as cellulose and hemicellulose in *A. gangetica*, which reduces CF digestibility. In this study, the CF digestibility was significantly higher ($p < 0.05$) in all the diets when compared solely with the *A. ganetica* diet (Table 3). This finding suggests that if the amounts

Table 3
Apparent nutrient digestibility and growth on rabbits fed with Asystasia gangetica *with different levels of corn*

Parameter	Treatment (mean ± standard deviation)				*p*-value
	T1	T2	T3	T4	
Digestibility (%)					
Dry matter	$88.8^{a} \pm 4.14$	$89.2^{a} \pm 3.00$	$81.2^{b} \pm 9.81$	$80.2^{b} \pm 2.74$	0.023
Organic matter	$73.0^{a} \pm 8.50$	$74.0^{a} \pm 7.69$	$49.8^{b} \pm 10.69$	$48.4^{b} \pm 5.14$	0.001
Crude protein	56.6 ± 20.49	55.2 ± 10.36	47.4 ± 9.67	56.9 ± 3.77	0.681
Ether extract	$82.0^{a} \pm 14.34$	$84.1^{a} \pm 8.70$	$49.6^{ab} \pm 32.15$	$32.7^{b} \pm 7.80$	0.005
Crude fibre	$43.8^{a} \pm 17.22$	$27.5^{ab} \pm 16.20$	$13.6^{b} \pm 11.41$	$7.7^{b} \pm 6.40$	0.012
Growth					
Initial BW (g)	1387.5 ± 205.6	1325.0 ± 119.0	1425.0 ± 132.3	1412.5 ± 154.8	0.757
Final BW (g)	1617.5 ±195.5	1476.7 ± 211.3	1482.5 ± 89.9	1412.5 ± 149.3	0.389
Total BW gain (g)	230.0 ± 57.2	176.7 ± 127.0	57.5 ± 168.6	0.0 ± 70.7	0.059
Daily BW gain (g)	8.2 ± 2.0	6.3 ± 4.5	2.1 ± 6.0	0.0 ± 2.6	0.059

Note. Means with different superscripts in a row differ significantly ($p < 0.05$); BW = Body weight

of CF intake by rabbits increase more than a certain amount of fibre, its digestibility tends to decrease. Hence, rabbits fed with the T4 diet demonstrate lower performance despite consuming relatively higher CP intake. This result coincides with the report by Mohammed et al. (2016), who found that high fibre content in diet could reduce the use of net efficiency of ME.

Although *A. gangetica* is a very nutritious plant, rabbits fed with *A. gangetica* did not show higher final body weight and weight gains. However, the energy and protein intakes were adequate compared to other treatments. It might be due to several factors: (i) improved fibre digestibility when the *A. gangetica* was supplemented with corn. In other words, the lower energy content of the *A. gangetica* (9.1 MJ/kg DM) relative to corn (12.9 MJ/kg DM) was compensated by improved digestibility of the energy, and specifically the fibrous fraction, (ii) the *A. gangetica* might have been of different quality when harvested, (iii) there might be an imbalance in mineral and vitamin intakes, as these two nutrients were not added in the diets, and (iv) some anti-nutritional factors present in the *A. gangetica* might have influenced the results. Nevertheless, since the duration of this study was too short (four weeks), it is still too early to make a solid assertion.

CONCLUSION

Rabbits fed *Asystasia gangetica* only demonstrated higher intakes/kg MW than those fed *A. gangetica* with corn. In contrast, the higher ME intake resulted in significantly ($p < 0.05$) higher digestibility values in rabbits fed with the highest level of corn supplement compared to those fed only *A. gangetica*. By using *A. gangetica ad libitum* with supplementation of different

levels of corn, farmers could improve the performance and productivity of rabbit rearing. Feeding *Asystasia gangetica* as a sole feed may not supply enough energy to support growth, although the protein in the forage may be sufficient. Nonetheless, further research with mineral and vitamin supplements using a larger sample size of animals for a longer period needs to be conducted to elucidate the findings reported in this study.

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Review Article

Advances and Future Prospects on Biotechnological Approaches Towards *Azolla* for Environmental Sustainability

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ABSTRACT

Environmental sustainability is an integral aspect of living a better life, which will continue to be globally highlighted in the future. Sustainable Development Goals (SDGs) are crucial in most research areas to improve natural resources that will ensure the long-term viability of the environment. The rising population may lead to increased pollution due to extensive anthropogenic activities. Natural resources are being increasingly exploited by an ever-increasing human population and rising per capita consumption. A combination of biotechnological approaches to strengthen environmental sustainability in plant fields has often been used. *Azolla*, an aquatic fern, is a promising candidate for worldwide application and is well established in biotechnology, particularly focusing on environmental sustainability. This review aims to explore the prospective of *Azolla* using a biotechnology approach. This review highlights current and future research and presents viewpoints on the importance of biotechnology in phytoremediation, genomics, and the animal feed industry.

Keywords: Aquatic fern, *Azolla*, biotechnology, environment, sustainability

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INTRODUCTION

Emerging technologies are increasingly marketed, ensuring long-term advantages along with growth in societies with environmental, economic, and socially responsible societies (Matthews et al., 2019). Biotechnology applications are a powerful strategy for assisting industries in achieving global sustainability, including textile processing (Rahman et al., 2020), animal feed (Kusmayadi et al., 2021), biofuel (Nasir et al., 2018), and agriculture (Adenle et al., 2012; Behera et al., 2021). With their high potential in different industries, it is necessary to use these technologies to aid in the implementation of better sustainability. According to Verma et al. (2011), biotechnology involves using living materials or biological products to produce current innovations for various applications, aiming to benefit humans. Environment biotechnology is helping manage the world's sustainability by reducing adverse effects. Furthermore, it brings forward existing research to be explored further to establish newer opportunities for environmental conservation.

Biotechnology is a fundamental aspect of achieving the Sustainable Development Goals (SDGs) of the United Nations, which will continue to emerge in the future as the global population expands. According to EuropaBio (2018), industrial biotechnology is linked to many SDGs, including SDG Goal 1: end poverty; SDG Goal 2: end hunger, achieve food security, and improve nutrition; SDG Goal 6: availability of water and sanitation sustainability; SDG Goal 7: ensure everyone access to cheap, dependable, and sustainable energy; SDG Goal 8: promote sustained, inclusive, and sustainable economic growth; SDG Goal 9: build resilient infrastructure and foster innovation; SDG Goal 11: make cities and human settlements inclusive, safe, resilient, and sustainable; SDG Goal 13: take immediate action to address climate change and its consequences; SDG Goal 14: conserve and sustainably use of the natural resources; and SDG Goal 17: re-establishing the global cooperation for sustainable development.

This review focused on an aquatic fern, *Azolla*, which has been exploited as an organic fertilizer to boost rice production in Southeast Asia for over 1,000 years (Lumpkin, 1980). It contributes to sudden global cooling by sequestering atmospheric carbon dioxide (Sessa et al., 2014). Despite its tiny size, it has various benefits for environmental conservation sustainably. The importance of the aquatic fern, *Azolla*, in ensuring ecosystem sustainability has long been established as soil fertilizer, bioremediation, and its part in mitigating greenhouse gas (Kollah et al., 2016). *Azolla* is not just a fern but also a superorganism (Li et al., 2014). Therefore, it has greater potential to achieve future sustainability goals defined by the United Nations through biotechnological approaches in a few main sectors, including animal feed, phytoremediation, and genomes (Figure 1). *Azolla*'s biotechnology approaches have been well established in a variety of studies, such as phytoremediation (Ghorbanzadeh

Mashkani, 2009; Goala et al., 2021; Talebi et al., 2019), enhancement of nutritive value (Brouwer et al., 2018; Costarelli et al. 2021), phylogenetic analysis (Metzgar, 2007; Reid et al., 2006), and cryopreservation (Brouwer et al., 2014).

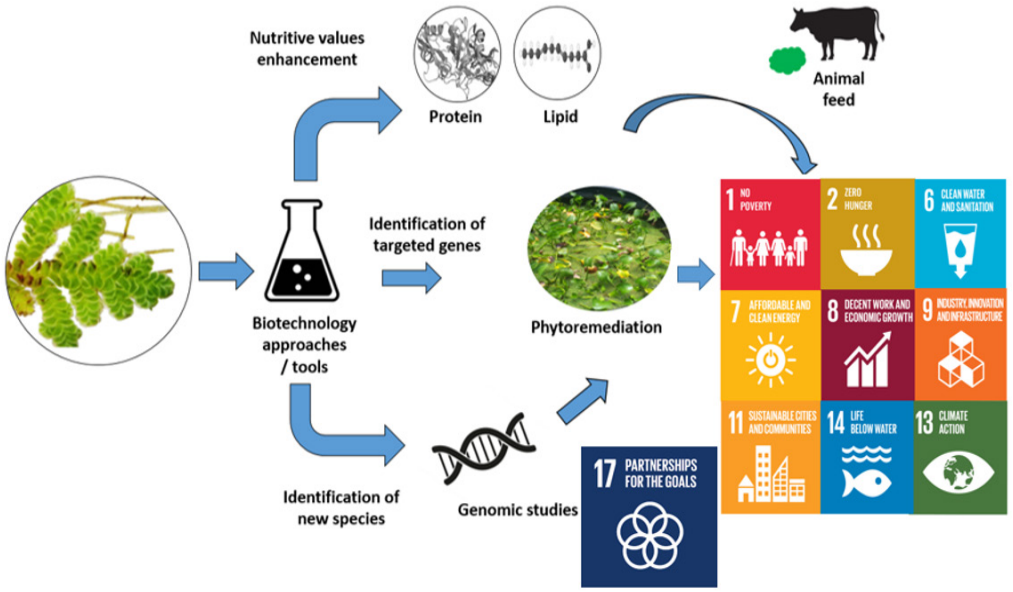

Figure 1. Biotechnological approaches in several sectors for *Azolla*

LITERATURE REVIEW

A literature search was conducted using Scopus, Web of Science, and PubMed databases with keywords of *Azolla* OR aquatic fern OR aquatic macrophytes AND biotechnology AND sustainability. The literature search was limited to a specific period, i.e., 2000–2021. It included research articles and a book chapter; only titles and abstracts were screened to remove irrelevant articles. This search also examined the reference list of the retrieved articles. Original research articles on the biotechnological application of *Azolla* published in English were included. The use of molecular tools to identify *Azolla* began in the early 90s, although its full genome was discovered in 2018. The first genomic data from ferns have been used to generate and understand the mechanisms that govern the evolution of plant genes and gene families (Li et al., 2014). According to PubMed, Google Scholar, and ScienceDirect databases, the total number of publications under the keywords of "biotechnology" and "*Azolla*" increased in 2020 compared with 1988 (Figure 2).

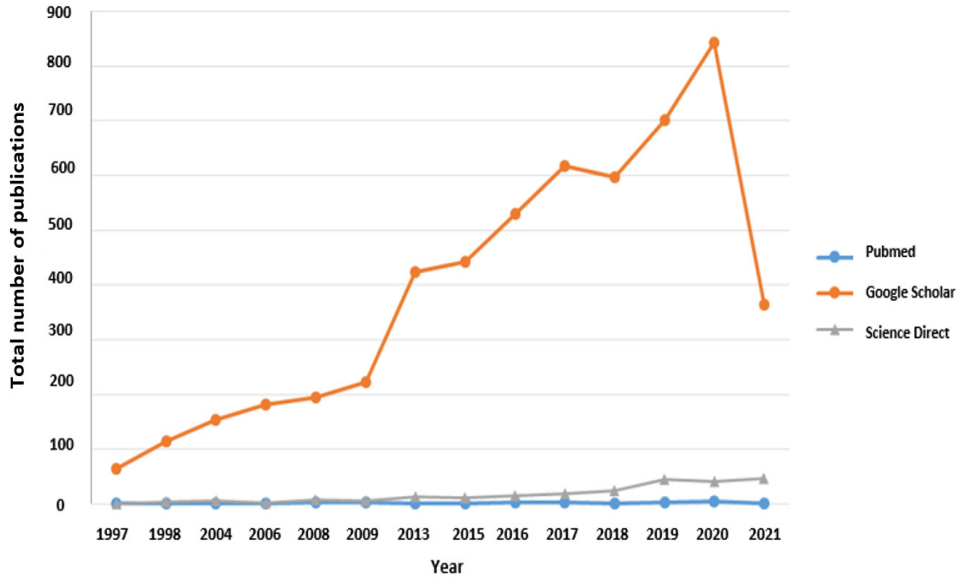

Figure 2. Research trends of biotechnology application in *Azolla* based on Science Direct, Google Scholar, and Pubmed databases

CURRENT STATUS OF BIOTECHNOLOGY IN *AZOLLA*

Azolla biotechnology approaches emerge rapidly and will continue to increase in the future. The biotechnology approach's effectiveness has mostly been focused on increasing the genetic diversity of *Azolla*'s species, phytoremediation, and improving nutritive value. This approach strategy had much success with this aquatic fern species. Additionally, one of the important aspects that have been considered in the development of molecular genetics, cellular, and genetic engineering, as well as genome editing, is to increase the efficiency in *Azolla*. Basic genetics and genetic engineering research also need to be utilised to increase the content of nutritive value and secondary metabolites in aquatic plants.

PHYTOREMEDIATION WITH BIOTECHNOLOGY TOOLS IN *AZOLLA*

Plants may be used to counteract the adverse effects of emissions and other human influences on the environment. The aquatic fern, *Azolla*, has an amazing ability as a phytoremediator to protect the environment from pollution (Naghipour et al., 2018). *Azolla* successfully detoxified some heavy metals, including arsenic (Zhang et al., 2008), ferrum, aluminium (Bianchi et al., 2020), plumbum, zinc, and cadmium (Khosravi et al., 2005). The genes related to phytoremediation in *Azolla* have been isolated and stated in Table 1 to understand the mechanism contributing to *Azolla*'s accumulation and detoxification effect. In a previous study, Schor-Fumbarov et al.

(2005) identified the efficiency of *Azolla* as a phytoremediator at the molecular level, where AzMT2 was linked to genes associated with *Azolla filiculoides*. The overexpression of phytoremediation-related genes in the molecular analysis may prove the effectiveness of plants as well-established phytoremediators (Nedjimi, 2021). According to Talebi et al. (2019), the expression of metallothionein and phytochelatin (MT2) and (PCS1), respectively, by *Azolla* was significantly induced by heavy metal treatment.

As part of the response to achieve environmental sustainability by reducing climate change, biochar is used to improve soil's physical, chemical, and biological properties, thereby reducing greenhouse gas emissions and sequestering carbon (Rawat et al., 2019). *Azolla* has been used as a primary organic source of biochar (Sadegh Kasmaei et al., 2019) along with other biochar sources (Dewi et al., 2018). Biochar is a high-carbon organic material produced via heat biomass without oxygen (pyrolysis) using biotechnology tools (Kimani et al., 2020). Table 1 lists several genes which are related to phytoremediation.

Table 1

Genes synthesized by Azolla *species related to different types of heavy metals*

Genes	Primers sequences	Heavy metals detected	Outcomes	*Azolla* species	References
Metallothionein-2 (MT2)	F GCAAGAGGAGCTTCGATGAGACC R CGCAAGAGCTATCGAACCCACAG	Copper (Cu), zinc (Zn), nickel (Ni), and cadmium (Cd)	Heavy metal treatments dramatically increased MT2 and PCS1 gene expression patterns	*Azolla filiculoides*	Talebi et al. (2019)
Phytochelatin synthase-1 (PCS1)	F TCCAATTCTCATCATTGCAGGAC R TCCAATTCTCATCATTGCAGGAC				
Actin	F TTGCTGATCGTATGAGCAAGGA R GATCCTCCAATCCAGACACTGTA				
nifB	5'-ATCCCTGCTACAGCGAAGAA-3' 5'-CCAGCAATTCCCAGAACTGT-3'	Zn, Cu, Cd, and Ni	Inhibit biomass growth in *Azolla*	*Azolla filiculoides*	Khosravi (2005)
	5'-ATGTCTTGCTGTGGAGGAAA-3' 5'-TTAGCATCGGCAAGGATT-3'				

GENOMIC STUDIES ON *AZOLLA* USING BIOTECHNOLOGY TOOLS

The complete genome sequence of *A. filiculoides* has been explored in a study by Li et al. (2018), which states that the genomic resources of *Azolla* are the most significant feature of future biotechnology research. Genetic diversity and phylogeny analysis for the identification of *Azolla* species was first established in 1993 by Van Coppenolle et al. (1993). Molecular tools are needed for the identification process in *Azolla* as their reproductive structure is rarely available. Although a vegetative structure can be used, it is unreliable (Madeira et al., 2016). Therefore, eliminating cyanobacteria from *Azolla* is the most crucial step for DNA extraction in molecular marker techniques. Cyanobiont (AzCy) was extracted from erythromycin-treated *A. filiculoides*, and its gene expression pattern was compared with that of the wild type (AzCy+) using several molecular techniques (Li et al., 2018). Using erythromycin, a type of antibiotic, before DNA extraction methods may improve the removal of cyanobacteria, thus allowing only the extraction of DNA from plants. Table 2 summarizes various molecular techniques for detecting *Azolla* and their DNA sequences.

Table 2

Molecular technique, DNA sequences, and the phylogenetic analysis in Azolla *species*

Molecular techniques	DNA fragments and sequences	*Azolla* species	References
Intergenic spacer	TrnL- (CGA AAT CGG TAG ACG CTA CG) trnF - (ATT TGA ACT GGT GAC ACG AG) trnLD - (GGG GAT AGA GGG ACT TGA A) trnLE - (GGT TCA AGT CCC TCT ATA CC)	*Azolla filiculoides*	Madeira et al. (2016)
	trnL-trnF region (the trnL intron and trnL-trnF intergenic spacer [IGS]), the atpB-rbcL IGS of the chloroplast, internal transcribed spacer (ITS) region of nuclear ribosomal DNA		Reid et al. (2006)
Random amplified polymorphic DNA (RAPD)	rbcL, atpB, rps4, and rps4-trnS	*Azolla pinnata* R. Br.	Chang et al. (2020)
	RAPD markers (OPA - OPF)		Pereira et al. (2011)

Table 2 *(Continue)*

Molecular techniques	DNA fragments and sequences	*Azolla* species	References
Sequence defined amplified region (SCAR) marker	F: GCCTAAGTCCAAGCTTACTCATCTTA R: ATTTAGGCTTAGGCCACAGATAGAAG F: CAATACCTTGTTCAGTGTTCGTAGG R: TGGCAATGTACCATGAAGTAGAATA F: AGATGGTTAGAAGTGACAGCATATCTTT RAT: TTCGC	*Azolla rubra*, *Azolla pinnata*, *Azolla filiculoides*, *Azolla microphylla*	Abraham et al. (2013)
Restriction fragment length polymorphism (RFLP)	CYA359F and 1051R	*Azolla rubra*, *Azolla microphylla*, *Azolla filiculoides*, *Azolla pinnata*	Sood et al. (2008)

The random amplified polymorphic DNA (RAPD) technique was performed using markers to identify *Azolla* species in the family of Azollaceae at the molecular level. Further, vegetative characteristics were used to identify *Azolla* species (Pereira et al., 2011). The findings revealed that the Shannon Index was higher (2.276) than vegetative characteristics (0.054), implying that molecular techniques are more reliable than morphological characteristics for species identification. Additionally, Dong et al. (2010) found greater genetic diversity in China's aquatic fern species *Ceratopteris*.

Additionally, the molecular approach of a specific sequence-defined amplified region (SCAR) was established using the nucleotide sequence of specific RAPD markers. According to Abraham et al. (2013), compared with RAPD, the SCAR approach has a slight advantage because RAPD markers detect many nonspecific fragments, whereas SCAR markers only identify specific RAPD fragments. Furthermore, the SCAR approach has been applied by Oyange et al. (2020) to evaluate the difference at the molecular level for a few *Azolla* species in Kenya.

Inter Simple Sequence Repeat (ISSR) markers have not been used in *Azolla* species, even though these approaches are commonly used in other aquatic fern species. Dong et al. (2007) applied the ISSR marker in *Ceratopteris pteredoides*, an aquatic fern species whose level of genetic diversity could provide valuable baseline data for conservation strategy. According to Wang et al. (2016), DNA barcoding is an emerging approach to identifying closely related fern species using short and standard sequences. As a method for species identification, DNA barcoding entailed the sequencing of a standard DNA region. According to this review, no studies on the DNA barcoding method were found for *Azolla* species.

BIOTECHNOLOGICAL APPLICATION OF *AZOLLA* FOR THE PRODUCTION OF ANIMAL FEED

As the human population is increasing worldwide, an alternative animal feed is one of the most important aspects to consider. According to Alemneh (2019), the role of biotechnology in the animal feed industry is crucial in two aspects, improving nutrient content in animal feed and production of animal feed. The recommended nutrient requirements for livestock are based on the evaluation by nutritionists (protein, amino acids, fatty acids, vitamins, and crude fibres) (Tona, 2018).

Several biotechnology-based methods were used to determine the nutritional value and phytochemical properties of *Azolla*, which livestock can consume. For example, the alkaline protein extraction method has been applied in *Azolla* and other aquatic plants of *Lemna*, *Spirodela*, and *Wolffia* to increase the crude protein levels by reducing condensed tannins (CTs) through protein precipitation (Brouwer et al., 2019). Further, the integrated biorefinery is one of the most recently developed methods to increase the amount of lipid, protein, and phenolic contents extracted from *Azolla* (Dohaei et al., 2020). Ultrasound-assisted water, sodium hydroxide extraction, and hot trichloroacetic acid extraction have been sequentially applied for protein extraction within an integrated biorefinery method. Advanced technology can also extract various primary nutritional values as part of the methodology. The technology advancements used in *Azolla* to improve the key nutritional values for animal feed production are listed in (Table 3).

Table 3

Several advanced technologies involve improving the main nutritive value in Azolla *species*

Technologies advancement	*Azolla* species	Nutritive value	Main findings	References
Elemental analyzer	*Azolla filiculoides*	Crude protein	20.6% of DW of protein content	Brouwer et al. (2019)
Fleurence method	*Azolla filiculoides*		12.6% of DW of protein content	Dohaei et al. (2020)
Kjeldahl method	*Azolla filiculoides*		12% of DW of protein content	Tran et al. (2020)
	Azolla pinnata		17% of DW of protein content	

Table 3 *(Continue)*

Technologies advancement	*Azolla* species	Nutritive value	Main findings	References
Acid hydrolysis using ion-exchange liquid chromatography	*Azolla filiculoides* and *Azolla pinnata*	Amino acid	The biomass's total amino acids (AA) ranged from 208 to 244 g kg^{-1} DW, and the AAs composed 82–88% of the total nitrogen.	Brouwer et al. (2018)
Ninhydrin colorimetric method	*Azolla pinnata*		39% higher in free amino acid levels as compared to control	Chen et al. (2017)
Gas chromatography-mass spectrometry GC-MS	*Azolla filiculoides* and *Azolla pinnata*	Fatty acids	Monounsaturated fatty acids (MUFA) make up just 10–12% of total FAMEs in *Azolla* species and are dominated by palmitoleic (C16:1) and oleic (C18:1) acids	Miranda et al. (2018)
High performance thin-layer chromatography (HPTLC)	*Azolla microphylla* and *Azolla caroliniana*	Vitamin (β – carotene)	471.73 mg/g in *A. microphylla* and 354.57mg/g in *A. caroliniana*	Azhar et al. (2018)

Note. DW = Dry weight

PROSPECTS OF BIOTECHNOLOGY IN *AZOLLA*

Further biotechnology application has shown *Azolla* has a unique feature of sustainable development through biotechnology, unlike any other higher plant. Its role in feeding the world's rapidly increasing population helps solve a significant issue for sustaining food security. A variety of biotechnological approaches can improve a plant's nutritional value and productivity. For example, the use of enzymes to boost the nutritional value and secondary metabolites can be introduced in *Azolla* plants. According to Le et al. (2016), methionine (MET) synthesis has been manipulated in genetic engineering research to boost MET content in plant proteins.

Using a bioreactor to cultivate *Azolla* can reduce the *Azolla*'s growth period and save cost. A study by Sobhani et al. (2020) showed that applying a low-cost disposable bioreactor shows the mass production of the *Hypericum perforatum* L. within four weeks. Duckweed is an aquatic plant with a strong potential for being produced on a large scale in a bioreactor (Coughlan et al., 2022; Yang et al., 2021). Moreover, the hybridization procedure should be performed to improve the valuable traits in *Azolla*.

Based on *Azolla's* increasing nutritional value yield, it can be declared as an alternative animal feedstock for livestock, aquaculture, and poultry to declare *Azolla* as an alternative animal feedstock for livestock, aquaculture, and poultry industries. Hemalatha et al. (2019) suggested that the structure of *Azolla*, which is a self-sustained closed-loop, tremendously benefits the environment. The use of alternative feedstock derived from aquatic plants and the adoption of biotechnology have been statistically demonstrated to aid in the sustainability of agricultural sectors. The effects of using *Azolla* as an animal feedstock for improving nutrient absorption efficiency in the gut metabolite system in the cattle and aquaculture industries should be further investigated. The complete genome sequences of *Azolla* species have provided several opportunities to investigate various phases and determine the genetic variation in *Azolla*'s species. DNA barcoding and cleaved amplified polymorphic sequence (CAPS) are the two molecular marker technologies that can be used in the future for *Azolla* species. Loss of genetic diversity in particular plant species is mostly due to environmental changes and demographic fluctuations in the short and long term (Khan et al., 2012). Therefore, this technology is critical for conserving the genomic DNA of uncommon and endangered plant species, particularly for aquatic water fern, *Azolla*, which is considered to have existed since the Carboniferous Period. The cost of handling the most significant hurdle to continuing integration of *Azolla* in several biotechnology techniques; therefore, *Azolla* should be widely employed in underprivileged nations. According to Lencucha et al. (2020), in developing countries, the government's responsibility is to provide farmers with incentives and support programs that are often needed to encourage sustainable agriculture practices.

CONCLUSION

Biotechnological approaches in *Azolla* have provided several opportunities for this unique aquatic fern to be further investigation of this unique aquatic fern for various environmental reasons. These approaches combine conservation programs, environmental sustainability, and food security systems under one tool. Several molecular techniques, such as intergenic spacer, RAPD, SCAR marker, and RFLP, have been explored, which can be further used in different *Azolla* species. However, more studies should investigate the current method in *Azolla* to better understand its role in the alternative for environmental sustainability, particularly as a phytoremediator, biopesticides, and biofertilizer to fulfil the increasing demand.

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Molecular Identification and Species Richness of Flies (Diptera) and Their Associated Bovidae Hosts at Cattle Farms in Selangor, Malaysia

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ABSTRACT

Flies (Diptera) play a significant role in the ecosystem as pollinators and decomposers, and they are also important vermin and disease vectors. Studies on the dipteran species are still lacking in Malaysia; therefore, the dipteran species' biology, morphology, distribution, and abundance are necessary. The objectives of this study were to identify dipteran species using a molecular approach, determine flies' Bovidae hosts, and investigate the diversity of the fly's species at three different cattle farms purposively selected in Selangor, Malaysia. The fly species were identified using cytochrome oxidase subunit I (*COI*) (*Haematopota javana*, *Tabanus rubidus*, *Tabanus fontinalis*, *Iranihindia martellata*, *Musca domestica*, and *Chrysomya megacephala*), while another six species only up to genus level (*Haematopota* sp. 1, *Musca* sp. 1, *Asilus* sp., *Metopia* sp., *Anasillomos* sp., and *Ommatius* sp.). In addition, two species of hosts: *Bos indicus* and *Bos taurus,* were proven to be the associated host species for the dipteran species based on molecular data of cytochrome b (*cytb*). However, there were no significant differences between farms in species diversity and richness ($F = 1.262$, $df = 2$, $p = 0.2459 > 0.05$). Interestingly,

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the two most dominant dipteran genera collected from the cattle farms were *Musca* and *Chrysomya*. At the same time, its abundance may have been influenced by the structure of the cattle cage flooring, which serves as a breeding site and food source. These findings contribute to fundamental epidemiological data in developing control strategies for dipteran species and are of great economic and health importance to livestock production in Malaysia.

Keywords: Blood-sucking insect, *COI*, *cytb*, DNA barcode, fly, host, livestock, Malaysia

INTRODUCTION

Members of the dipteran species (Diptera) are commonly referred to as true flies or two-winged flies and comprise black flies, fruit flies, horse flies, house flies, midges, and mosquitoes. It is among the most diverse insect orders, with an estimation of 120,000 to 150,000 species (Brown, 2001; Colless & McAlpine, 1991; Courtney et al., 2017; Schumann, 1992). The diversity of the dipteran group is evident not only in their species richness but also in their diverse habits and habitats, as well as their significant effect on agriculture, forestry, animal, and human health (Courtney et al., 2017; Skevington & Dang, 2002; Ssymank et al., 2008).

Diptera is one of the most important insect orders in terms of their interaction with humans, especially in spreading diseases and causing agricultural losses (Courtney et al., 2017; Marshall, 2012; Pape, 2009). However, Diptera's benefits to the ecosystem are also significant, albeit less understood. For example, flies contribute to plants' pollination, biological control of pests, and degrade dung, carrion, and other organic matter (Marshall, 2012; Skevington & Dang, 2002). Previous researchers like Marshall (2012) and Pape (2009) have emphasised the study of this diverse group of insects on their impact on humans and their active role in ecosystem functions.

As a primary group in diverse ecosystems and environments, these pestiferous insects significantly impact animal and human health, agriculture, and forensic sciences (Singh & Bharti, 2000). Their close association with humans has led them to be recognised as important vermin and disease vectors, and some flies are responsible for a multitude of illnesses and deaths inflicted by humans worldwide. However, flies also play beneficial roles as key components in many diverse ecosystems (Skevington & Dang, 2002). It is worth noting that studies and reports on Diptera are still lacking in Malaysia compared to other parts of the world, where substantial studies have been documented on the biology, morphology, distribution, and abundance of the dipteran species (Gerhardt & Hribar, 2019; Marshall, 2012).

Morphological identification is a traditional taxonomic tool where a species is identified based on the morphological characters and by referring to the description of the identification keys. Morphological identification is still the preferred method for research as it requires little technical equipment, is easy to implement in the

field, and is relatively cheap even when large numbers of individuals need to be identified. However, the limitations in using morphology-based identification include being tedious, time-consuming, and difficult to distinguish cryptic species. In addition, the dwindling number of skilled and experienced taxonomists globally has triggered critical problems in identifying species based on morphology alone (Renaud et al., 2012).

Molecular identification methods are faster and have increased sensitivity; thus, they are now being advocated for identification. Accurate identification of dipteran species in livestock farms is very important for determining their role in disease transmission and planning effective vector control and management strategies. Although dipteran species identification based on morphological features is economical, easy to perform, and requires no complicated equipment, this methodology requires the skills of experienced taxonomists. In addition, the specimens need to have clear external morphological characteristics, involving proper specimen preparation. Misidentification would adversely affect the efficacy of vector control and influence the control and preventive measures in disease transmission. Therefore, molecular-based identification can be used to solve identification ambiguities in morphologically similar species, as well as in species lacking important morphological traits or specimens in immature life stages.

DNA barcoding is a molecular method that uses a short fragment of the nucleotide of a specific gene for accurate species identification. DNA barcoding has gained wide attention and plays an important role in precise species identification and in revealing genetic diversity in the presence of any biotypes, haplotypes, and/or genotypes, with the power to resolve taxonomic ambiguities at the species level and within species complexes. The barcoding region of mitochondrial cytochrome oxidase subunit I (*COI*) has been shown to effectively discriminate species in a range of dipterans including tabanids (Changbunjong et al., 2018; Cywinska et al., 2010; Morita et al., 2016). *COI* is a mitochondrial gene commonly used to support morphological identification by amplifying a region of the gene using a set of universal primers during polymerase chain reaction (PCR) (Banarjee et al., 2015). Advances in molecular techniques for blood meal analysis and barcoding using PCR-based assays and direct sequencing of the cytochrome b gene (*cytb*) have permitted the identification of hosts with a higher degree of accuracy compared to previous serological techniques (Alcaide et al., 2009; Molaei et al., 2008; Townzen et al., 2008).

One of the significant problems in livestock farms all over Malaysia is the fly menace as disease vectors for human and animal health reported on several stable and horse fly species vectoring *Trypanosoma evansi* that lead to the trypanosomiasis outbreak in Malaysian ruminants such as buffalo, cattle, and deer that cause severe losses in body weight and milk production (Erwanas et al., 2015). Documented literature

on Dipterans in local livestock farms is still considerably sparse despite this group's significance and economic importance as pests and disease vectors. Current studies on host identification of Dipteran species in Malaysia have various technical and related constraints. Therefore, the objectives of this study were to molecularly identify the fly species at several cattle farms in Selangor, Malaysia: to determine the mammalian host for the fly's species molecularly and to determine the flies' abundance and richness from three different farms with different structures of cages flooring.

METHODS

Sampling Sites

This study was conducted on three different livestock farms in Selangor: Ladang Bangi (2°55'44.6"N 101°46'31.6"E), Ladang Rasa (3°29'32.4"N 101°37'31.6"E), and Ladang 16, UPM (3°0'26"N101°42'16"E). The livestock farms in Selangor were sampled as the model farms to determine the richness of dipteran species in Peninsular Malaysia. The selection of the cattle farm in this study was purposively made based on the building of the cage structure and the surrounding area. For Ladang Bangi, the cattle cage had ground flooring and was adjacent to the fragmented forest ecosystem and a river. Meanwhile, for Ladang Rasa, the cattle cages were partially covered with ground and concrete flooring, while the farm was adjacent to the fragmented forest ecosystem. As for Ladang 16, UPM, the cage was built from concrete flooring, and the farm was in the open area of the UPM campus and connected to the main road.

Sampling Methods

Passive Sampling. Six baited traps were placed randomly in the cages in each of three selected livestock farms in Selangor from May 2019 to August 2019. The bait trap is a useful method to study the fly populations. A mixture of chicken liver and cow urine was used as bait for each trap. Four visits were made to each livestock farm during the sampling period. Six traps were left randomly for a week in the field. Afterward, the fly specimens were collected in a bottle containing 100% alcohol for wet preservation and taken back to the laboratory for identification and molecular analysis. The specimens were sorted based on external morphology and stored in vials containing 100% alcohol, each vial with a label indicating sampling locality and collection date of the specimens.

Active Sampling. Flies were collected from the bodies of their host cattle in the cage areas using a sweeping net. During every visit, the cattle were kept inside the cowshed on all the farms throughout the sampling period. The samples were collected in a bottle containing 100% alcohol and immediately brought back to the laboratory for sorting based on external morphology. The specimens were stored in 100% alcohol for wet preservation vials, with labels indicating locality and collection date. Before molecular analysis, these specimens were stored in the freezer at -20°C.

Laboratory Works

Morphological Identification of Dipteran Species. The specimens collected were examined using a stereo microscope (Zeiss Stemi DV4, Germany). Several taxonomic keys and species descriptions were utilised to identify the specimens (Al-Talafha et al., 2017a, 2017b; Kurahashi et al., 1997; Nihei & de Carvalho, 2009), which were mainly based on external morphology characteristics (eyes, antennae, wing venation, thorax, and abdominal pattern and colour, as well as body length). Images of the specimens were taken using a camera (Canon EOS1000D, Japan). Molecular identification was then utilised to reconfirm the morphological-based identification.

DNA Barcoding Analysis. The *COI* region was PCR-amplified to identify the flies at the species level to support the morphological identification of the flies. The five main processes of molecular methods of DNA barcoding performed were DNA extraction, polymerase chain reaction (PCR), purification of PCR products, DNA sequencing, and data analysis.

DNA Extraction. DNA from individual fly specimens per species or morphospecies was extracted from the whole body using the NucleoSpin® DNA Insect (Machery-Nagel, Germany) extraction kit. The samples were soaked in ATL buffer and proteinase K for lyses, according to the manufacturer's instructions (Halim et al., 2017, 2018), and stored at -4 °C for further molecular work.

Polymerase Chain Reaction (PCR) and DNA Purification. DNA extracted from different fly samples was amplified using the GeneAmp PCR System 2400 (Perkin Elmer, USA) and cytochrome oxidase subunit I (*COI*) mitochondrial DNA based on Hebert et al. (2003) and Shariff et al. (2014) profiles. The amplification product of forwarding primer, COI- LCO1490 5'GGT CAA CAA ATC ATA AAG ATA TTG G 3' and reverse primer COI- HCO22198 5'TAA ACT TCA GGG TGA CCA AAA AAT CA 3' was 750 bp, in reference to Folmer et al. (1994). The PCR parameter for amplification of *COI* gene consisted of pre-heating 94 °C (60 sec), initial denaturation, 94 °C (60 sec) for 5 cycles, cooling 45 °C (45 sec), extension 72 °C (90 sec), denaturation 94 °C (45 sec) for 30 cycles, cooling 52.5 °C (75 sec), extension, 72 °C (75 sec), final extension, 72 °C (300 sec), and storage/holding, 4 °C (∞).

The same DNA extracted from the flies was used for host identification. PCR with universal animal primers targeting the cytochrome b gene (*cytb*) was used to detect the flies' host. The primers used for host identification of dipteran species were according to Kocher et al. (1989). Primers 5'-3' *cytb* L14841 5' AAAAAGCTTCCATCCAACATCTCAGC ATGATAA 3' and H15149 5'AAACTGCAGCCCCTCAGAATGATAT TTGTCCTCA 3' produced an amplicon of around 500 bp. The PCR parameters consisted of pre-heating 95 °C (180 sec), initial denaturation 95 °C (15 sec), cooling 51.8 °C (30 sec), extension 72 °C (10 sec), denaturation 95 °C (15 sec) for 30 cycles,

cooling 51.8 °C (30 sec), extension 72 °C (10 sec), final extension 72 °C (600 sec), and storage at 4 °C (∞). PCR products were purified using GF-I PCRCLEAN-UP Kit (Vivantis, United Kingdom) to remove excess dNTP and buffer. The purification procedure was conducted based on the manufacturer's instructions. The PCR products were electrophoresed for 30 minutes at 90V and 1.5% agarose gel and photographed under UV light. Images were captured with a gel imager AlphaImager HP (Alpha Innotech, USA).

DNA Sequencing Analysis and Sequences Alignment

PCR products with clear bands after electrophoresis along with the forward and reverse primers were sent for sequencing at Apical Scientific Sdn. Bhd. (Seri Kembangan, Malaysia). DNA sequences were edited using BioEdit Sequence Alignment Editor (BioEdit v7.0.5) to obtain accurate sequences. The edited sequences were then used as the input in the Basic Local Alignment Search Tool (BLAST) for molecular identification. The species separation presented was based on the Neighbour-joining (NJ) analysis using Phylogenetic Analysis Using Parsimony* (PAUP* v4.0b10). The NJ tree was constructed based on the Kimura-2 parameter (K2P) model and bootstrap with 1,000 replications.

Species Abundance and Composition

The total number of individuals and species collected from all localities was recorded to determine the species composition and abundance and is expressed as a percentage. Parameters of the diversity indices were calculated using Shannon-Weiner Index (*H'*), Margalef Index (*D*), and Evenness Index (*E*). One-way analysis of variance (ANOVA) was conducted to measure the significance of diversity between farms. Paleontological statistics software for education and data analysis (PAST 4.03) (Hammer et al., 2001) was used to analyse the diversity data.

RESULTS

Morphological Identification of Dipteran Species

Four dipteran species were successfully identified morphologically at the species level - *Haematopota javana*, *Tabanus rubidus*, *Tabanus fontinalis*, and *Musca domestica*. Meanwhile, nine species were successfully identified up to the genus level: *Sarcophaga* sp., *Iranihindia* sp., *Haematopota* sp. 1, *Musca* sp. 1 *Chrysomya* sp., *Asilus* sp., *Metopia* sp., *Anasillomos* sp., and *Ommatius* sp. Finally, all the species and morphospecies were subjected to molecular barcoding to re-confirm the species identification (Table 1, Figure 1).

Molecular Identification of the Dipteran Species

A representative from each species and morphospecies underwent the DNA barcoding process. The BLAST analysis indicated a high similarity percentage (>97%) for *Chrysomya megacephala* (99.86%), *Tabanus fontinalis* (99.85%),

Table 1
Species composition, abundance, and richness of the collected samples from three farms

Species	Number of individuals collected		
	Ladang Bangi	Ladang Rasa	Ladang UPM
Anasillomos sp.	2	0	0
Asilus sp.	1	0	0
Chrysomya megacephala	0	0	40
Haematopota javana,	0	3	0
Haematopota sp. 1.	1	0	0
Iranihindia martellata	0	0	0
Metopia sp.	0	4	0
Musca domestica	0	14	21
Musca sp. 1	0	0	9
Ommatius sp.	5	0	0
Sarcophaga sp.	4	0	0
Tabanus rubidus	1	3	0
Tabanus fontinalis	0	1	0
Number of individuals	16	25	70
Number of species (N)	6	5	3
Margalef Index (*D*)	1.895	1.259	0.4708
Evenness Index (*E*)	0.8004	0.6819	0.8574
Shannon-Weiner Index (*H'*)	1.569	1.227	0.9447

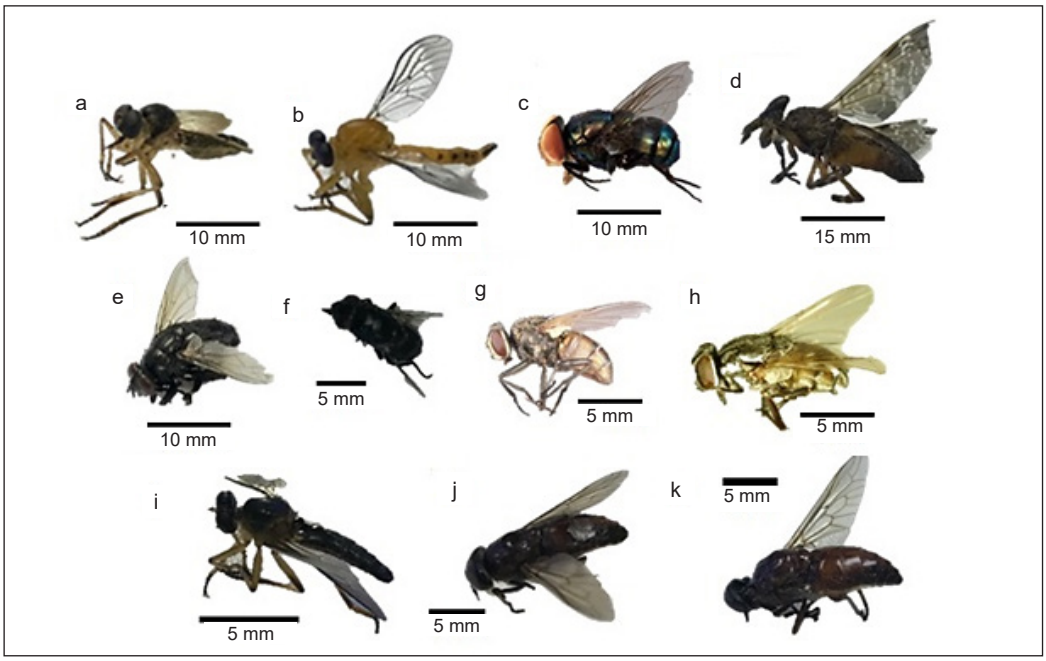

Figure 1. Dipteran species successfully collected and used for molecular analysis in this study: a. *Anasillomos* sp.; b. *Asilus* sp.; c. *Chrysomya megacephala*; d. *Haematopota javana*; e. *Iranihindia martellata*; f. *Metopia* sp.; g. *Musca domestica*; h. *Musca* sp. 1; i. *Ommatius* sp.; j. *Tabanus fontinalis*; k. *Tabanus rubidus*

Tabanus rubidus (99.09%), *Iranihindia martellata* (99.29%), *Haematopota javana,* (98.59%), *Haematopota* sp. (97.72%), *Musca domestica* (99.57%), but low similarity percentage (<97%) for *Musca* sp. (94.63%), *Anasillomos* sp. (93.02%), *Ommatius* sp. (89.97%), *Metopia* sp. (90.0%), and *Asilus* sp. (85.11%) (Table 2). Species separation was visualised in the NJ tree (Figure 2).

Identification of the Associated Mammalian Host Species

Two host mammal species were successfully detected from the DNA of the dipteran samples collected from four localities: *Bos indicus* and *B. taurus*. Based on the BLAST results, *B. indicus* and *B. taurus* showed a high similarity percentage (>98%) in all species, except 98.33% in *B. taurus* (Table 2). However, several species were not successful PCR-amplified, while others had contamination.

Species Composition and Abundance

A total of 111 dipterans were successfully collected from three farms and four livestock localities in Selangor, Malaysia, and comprised nine genera in five families. The dipteran flies collected from all study sites consisted of five families: Muscidae, Tabanidae, Asilidae, Sarcophagidae, and Calliphoridae. The dipteran genera collected in this study were *Musca* (40%), *Chrysomya* (37%), *Ommatius* and *Sarcophaga* (4%), *Haematopota, Tabanus*, *Metopia* and *Iranihindia* (3%), *Anasillomos* (2%), and *Asilus* (1%). The genus *Musca* recorded the highest abundance, with 44 individuals or 40% of the overall Diptera collected, followed by *Chrysomya* with 40 individuals (37%), *Ommatius* and *Sarcophaga*, with five individuals (4%), respectively. The genus *Asilus* recorded the lowest abundance, with only one individual or 1% of the overall Diptera collected (Figure 3). Ladang Bangi presented the highest Shannon-Weiner Index (*H'*) and Margalef Index (*D*), followed by Ladang Rasa, and lastly, Ladang UPM, with 1.569 (1.895), 1.227 (1.259), and 0.9447 (0.4708), respectively. One-way ANOVA with ($F = 1.262$, $df = 2$, $p = 0.2459 > 0.05$) showed no significant difference between farms in species diversity and species richness.

DISCUSSION

The various dipteran families of Muscidae, Sarcophagidae, Asilidae, Calliphoridae, and Tabanidae, were sampled in the field to gather information on the species richness and abundance. Although previous studies have been conducted on these families, they focused more on the diversity of a specific family, genus, and species in Malaysia (Khofar et al., 2019; Phasuk et al., 2011; Ya'cob et al., 2020). Three farms were purposively selected in this study as the sampling sites in Selangor to serve as model farms to represent other parts of Peninsular Malaysia. This assumption is based on earlier study findings and the rationale that the dipteran species are randomly distributed throughout Peninsular Malaysia, mainly along the altitudinal gradient (Ya'cob et al., 2016).

Table 2
Dipteran species and their host species were identified by using molecular data, the similarity percentage, and the accession number

Sample code	Locality	Fly species (from the Genbank)	Similarity percentage (BLAST)	Fly species (Accession no.)	Host species (from the Genbank)	Similarity percentage (BLAST)	Host species (Accession no.)
SBJ 1	West Malaysia: Selangor: Ladang Bangi	*Haematopota javana*	97.72% (e-value = 0.0)	*Haematopota* sp. 1 (MZ769394)	*Bos indicus*	99.38% (e-value = 0.0)	*Bos indicus* (MZ851350)
SBJ 3		*Ommatius* sp.	89.97% (e-value = 0.0)	*Ommatius* sp. (MZ769388)	-	-	-
SBJ 4		*Tabanus rubidus*	99.09% (e-value = 0.0)	*Tabanus rubidus* (MZ769389)	*Bos indicus*	99.56 % (e-value = 0.0)	*Bos indicus* (MZ851349)
SBJ 5		*Anasillomos* sp.	93.02% (e-value = 0.0)	*Anasillomos* sp. (MZ769390)	-	-	-
SBJ 6		*Asilus sericeus*	85.11% (e-value = 0.0)	*Asilus* sp. (MZ769391)	-	-	-
SBJ 7		*Iranihindia martellata*	99.29% (e-value = 0.0)	*Iranihindia martellata* (MZ769392)	*Bos taurus*	98.33% (e-value = 0.0)	*Bos taurus* (MZ851356)
SR 1	West Malaysia: Selangor, Ladang Rasa	*Haematopota javana*	98.59% (e-value = 8e-176)	*Haematopota javana* (MZ769393)	*Bos indicus*	99.79% (e-value = 0.0)	*Bos indicus* (MZ851351)
SR 4		*Metopia campestris*	90.00% (e-value = 0.0)	*Metopia* sp. 1 (MZ769384)	*Bos indicus*	99.79% (e-value = 0.0)	*Bos indicus* (MZ851352)
SR 3		*Musca domestica*	99.57% (e-value = 0.0)	*Musca domestica* (MZ769386)	*Bos indicus*	98.35% (e-value = 0.0)	*Bos indicus* (MZ851353)
SR 5		*Tabanus fontinalis*	99.85% (e-value = 0.0)	*Tabanus fontinalis* (MZ769395)	*Bos indicus*	99.79 %. (e-value = 0.0)	*Bos indicus* (MZ851354)
SR2		*Metopia campestris*	90.00% 8996 (e-value = 0.0)	*Metopia* sp. (MZ769385)	-	-	-
L1	West Malaysia: Selangor: Ladang UPM	*Chrysomya megacephala*	99.86% (e-value = 0.0)	*Chrysomya megacephala* (MZ769396)	*Bos indicus*	98.96% (e-value = 0.0)	*Bos indicus* (MZ851355)
L2		*Musca convexifrons*	94.63% (e-value = 0.0)	*Musca* sp. 1 (MZ769387)	-	-	

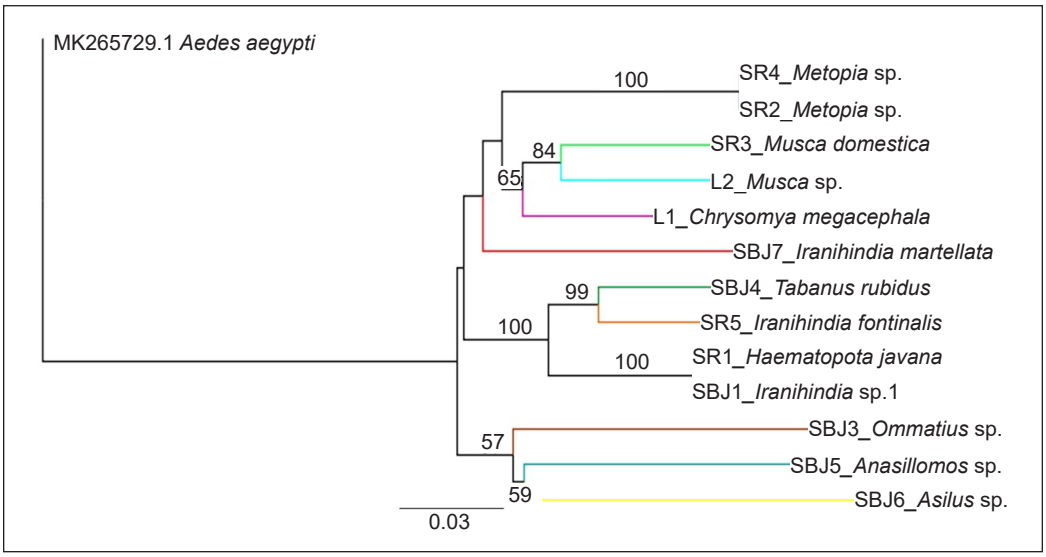

Figure 2. Neighbor-joining tree implemented for all the barcoded flies' species with 1,000 bootstrap replications

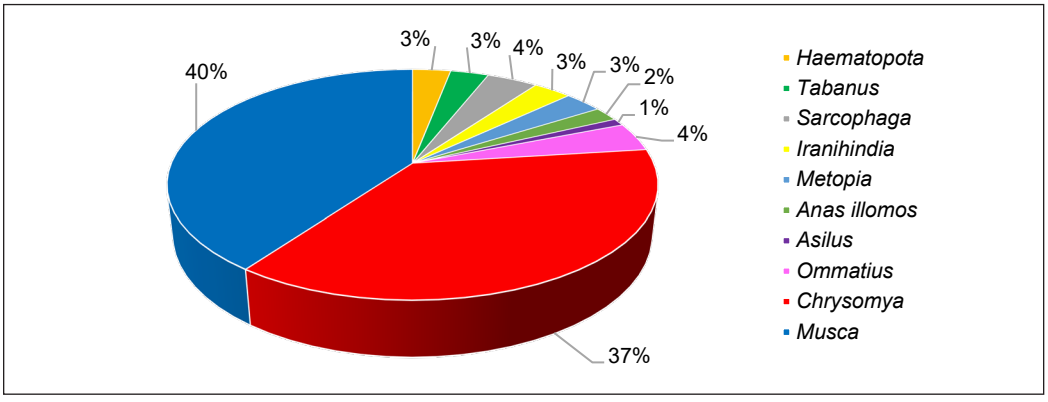

Figure 3. Composition of dipteran species according to genera collected from three cattle farms in Selangor

Identification of species based on morphological characteristics is a conventional method in the insect identification process. However, the lack of morphological characteristics has limited their efficiency and accuracy (Tahir et al., 2018). Based on the available keys and taxonomic references, only four species could be identified up to the species level. In contrast, some morphospecies of the genus *Haematopota* sp. 1, *Ommatius* sp., *Anasillomos* sp., *Asilus* sp., and *Metopia* sp. could not be identified at the species level due to a lack of available taxonomic keys. Therefore, the problematic species were barcoded for precise identification due to ambiguities in species identification.

The barcoding analysis was conducted using the *COI* marker, which is a highly useful molecular tool that can discern cryptic, closely related, and morphologically similar species (Clark et al., 2005; Della Torre et al., 2002) and has been successfully utilised for species identification in various organisms (Rivera & Currie, 2009). In addition, DNA barcoding is also very valuable in species reconfirmation during the immature stages of insects (Barrett & Hebert, 2005). All the sequences obtained in this study showed more than 84% similarity to the Genbank sequences. In 13 sequences, 7/13 sequences showed low species detection (84-97%), while 6/13 sequences were considerably high (>97% similarity). According to Morinière et al. (2019), the sequences that showed more than 82% similarity to Genbank could be confirmed as genus level, while >97% similarity with Genbank would prove that the species in question was the right species. Failure to identify up to the species level by molecular approach might be due to the DNA quality. However, we thought this would not be the major issue because the 5–10ng/µl of DNA concentration in insect species can be amplified successfully. The newly presented *COI* sequences not yet available in the Genbank may cause a lower percentage with the Genbank similarity (Molaei et al., 2008).

Two dipteran genera, i.e., *Musca* and *Chrysomya* sp., were the most abundant in the cattle farms, and our results confirmed those of Al-Shaibani and Al-Mahdi (2014), who reported that *Musca* was the most abundant genus in Yemen animal farms and functioned as the main decomposer of cattle dung (Hussein et al., 2017) since various species under the genus *Musca* were ubiquitous, and highly adaptive to different ecosystems, even under extreme environmental conditions. However, this housefly species is a great nuisance and pest to humans and livestock, causing reduced livestock productivity as a disease carrier and vector of many pathogens, with an economic loss estimated at billions of dollars annually (Khamesipour et al., 2018; Taylor et al., 2012).

The blowfly or oriental latrine fly *Chrysomya megacephala*, which belongs to the family Calliphoridae, has significant importance in environmental health as a decomposer of faeces and decaying carrion, as well as in forensic entomology (Mullens, 2009; Sharanya & Zuha, 2019). For this reason, the species may breed prolifically on cattle farms and in human settlements. In addition, both the housefly and the blowfly species had been reported to have the potential to carry pathogenic bacteria and were abundant in human settlements in Northeast Thailand (Chaiwong et al., 2014).

Ladang Bangi farm showed the highest number of fly diversity, while Ladang UPM farm showed the lowest. The fly species diversity and richness appeared to be associated with the conditions of the farm, such as the structure of the dairy building and the type of cage flooring (Lysyk & Axtell, 1986). Our results indicated that high infestations of *Musca* spp. and *Chrysomya* sp. were recorded in Ladang UPM, mainly

due to the sanitation status or manure management around the cages, where both groups of fly species utilised cattle dung as their breeding sites (Khan et al., 2012). The sanitation status refers to the type of cage flooring, whether concrete, half concrete, or ground flooring, as a medium or surface which affects the decomposition rate of the manure or its natural disposal in the environment.

The high presence of *Chrysomya* sp. (besides *Musca* spp.) on Ladang UPM farm was due to the behaviour of this species which sought cattle dung as its preferred food source, as well as its breeding site (Wang et al., 2018). Infestation of fly larvae in live animals can cause myiasis, leading to tissue necrosis in cattle (Ferraz et al., 2010), which was not recorded in our study area. Therefore, we surmised that the Ladang UPM farm might need further judicious sanitary management, such as disposing of the cattle dung twice during the peak breeding period of the flies, together with a single or combined application of pesticide to overcome the infestation problem (Issa, 2019). However, both *Musca* spp. and *Chrysomya* sp. were absent at Ladang Bangi, likely because the earthen flooring of the cages was not a conducive breeding site for the flies, besides the lack of food source, i.e., the faeces, which had dried out and dissipated into the farmlands. Ladang Rasa farm recorded the presence of *Musca* sp., but not *Chrysomya* sp., most likely due to the combined structure of the ground and cement flooring of the cages, favouring *Musca* sp. infestation (Issa, 2019; Lysyk & Axtell, 1986). Comparing the three farms, the Ladang Bangi farm provided the highest number of fly species diversity, followed by Ladang Rasa and Ladang UPM, respectively. Both farms (Ladang Bangi and Rasa) had a high diversity of fly species probably because both were adjacent to the fragmented forest, which could provide suitable niches for different fly species. The Ladang Bangi farm recorded the highest fly diversity, most likely because of its proximity to a river, as well as the fragmented forest, and thus, could provide more microhabitats for a wider variety of fly species and their hosts (Brockerhoff et al., 2017). Ladang UPM farm presented the lowest fly species diversity and richness, probably due to the farm being on open ground and adjacent to the main road, thus, subjected to anthropogenic factors which could affect the breeding sites and food sources of the various fly species (Papanastasis et al., 2017).

The depredation of blood-sucking and myiasis-producing flies is detrimental to the productivity and profitability of animal husbandry worldwide (Gerhardt & Hribar, 2019). In this study, *Tabanus* sp. from the family Tabanidae was present on the cattle farm but did not feed on human blood. However, there were records of several species under the genus *Tabanus*, namely *Tabanus bromius* and *Tabanus distinguendus,* that sucked human blood, causing human granulocytic anaplasmosis (HGA) by transmitting the pathogen *Anaplasma phagocytophilum* (Werszko et al., 2019). Their attack can lead to

weight loss and reduced milk production in livestock, and they can transmit several disease pathogens, including protozoa, bacteria, and viruses (Baldacchino et al., 2014; Foil, 1989; Mullens, 2009).

Furthermore, *Haematopota javana* was also found in the cattle farms in this study, but the family richness of Tabanidae in this study was considered low compared to a previous study by Phasuk et al. (2011). Only three species of tabanids were collected in our study compared to 10 species by Phasuk et al. (2011). However, our findings were not directly comparable because of differences in methodology and study duration (i.e., their sampling method utilised malaise traps for 18 months versus our sampling method used baited traps and sweeping nets for three months). Furthermore, the abundance of tabanids is also significantly higher during the wet season in Thailand, but this seasonal variation was not apparent during our sampling period in the field.

Notably, the black fly species *Simulium* sp. (family Simuliidae) was not found in this study, although it has been recorded as a vector that transmits pathogens in livestock farms. According to Adler et al. (2010), this species is prevalent in the stream area and causes the parasitic disease known as onchocerciasis or river blindness in livestock and humans. Four sarcophagids (also known as flesh flies) species, i.e., *Sarcophaga* sp., *Metopia* sp. 1, *Metopia* sp. 2, and *Iranihindia martellata,* were reported in this study. Most sarcophagid flies can cause myiasis (invasion of tissues and organs in humans and animals by the larvae of the saprophagous flies). These larvae feed on the host tissues and body fluids or ingest food as parasites in the skin, many body parts, and other soft tissues of humans (Hall et al., 2016).

Anasillomos sp., *Asilus* sp., and *Ommatius* sp. are classified under Asilidae, also known as the robber fly. The asilid adults are predators capable of taking on larger prey such as dragonflies, but the selected prey size varies among the species. The type of prey, whether stationary, crawling, or flying, is also species-specific among the robber flies. Their mouthparts contain a stout proboscis that the adult uses to inject paralysing venom into the prey during hunting. Asilids are not blood-feeders, but their bites can cause pain to humans when confronted or disturbed (Newton, 2006).

Eight sequences under six dipteran species were detected carrying the DNA of the host cattle, as confirmed through PCR and DNA sequencing. The positive and negative controls supported the results in each PCR process (Banasik et al., 2016). The main idea was to detect the small amount of DNA of the host species in the flies' DNA using the targeted animal species primers. Identification of the host species was obtained with >98% match for *B. indicus*, except for 98.33% similarity with the Genbank for *B. taurus*. In the host preference analysis using *cytb*, *B. taurus* and *B. indicus* were successfully detected in the DNA of the fly species. Both cattle species are important for meat and dairy products and have higher productivity from

the breeding process with selective local and pure breeds (International Atomic Energy Agency [IAEA], 2009). Furthermore, DNA of *B. taurus* was detected in the sucking flies, namely *H. javana*, *T. rubidus*, and *T. fontinalis*, which might act as potential vectors of pathogens causing Surra disease or trypanosomiasis in cattle, as reported in India (Veer, 1999) and in Malaysia (Erwanas et al., 2015).

Using the molecular approach, several flies in this study were associated with the cattle species, namely *Sarcophaga* sp., *Iranihindia martellata*, *Musca domestica*, and *Musca* sp. 1. These novel findings were proven using molecular approaches to detecting the DNA of the cattle species (either *B. taurus* or *B. indicus*) from the DNA of the dipteran species. The detected fly species were categorised under Sarcophagidae and Muscidae, which play a significant role as flesh flies and mechanical vectors of pathogens. Furthermore, the DNA of the cattle species was found in the bodies of the flies that had visited the carrion of the cattle species (Patton, 1922; Sukontason et al., 2014; Tan et al., 2010).

Identifying meal with DNA barcoding using the *cytb* gene was essential to determine the insect vector's host range and host preference. Although this can also be achieved using previous serological techniques (Alcaide et al., 2009), the PCR-based method enabled the identification of hosts up to the species level with a much higher degree of accurate identification, indicating >95% similarity with Genbank (Molaei et al., 2008; Townzen et al., 2008). Species confirmation of the flies and their associated hosts is important in studying pathogen transmission and determining potential biological and mechanical vectors of infectious pathogens.

Bovidae hosts could not be detected in several fly samples via PCR amplification, probably due to the lack of cattle DNA inside the dipteran samples and human contamination. The DNA of the potential Bovidae host can be amplified even in very low quantities and by designing specific Bovidae primers (Lee et al., 2015). The utilisation of gBlock primers is necessary to avoid human contamination (Boessenkool et al., 2012).

The effectiveness of DNA sequence detection in barcode analysis was highlighted by Ernieenor et al. (2015) in ticks and by Slama et al. (2015) in their blood meal analysis of the blood-sucking *Culicoides* (Diptera: Ceratopogonidae). Furthermore, the pathogens carried by the dipteran species can also be detected by using different pairs of primers targeting the pathogens, as reported by Hemmatinezhad et al. (2015).

CONCLUSION

The new barcodes of 11 dipteran species successfully identified in this study were deposited in Genbank, as well as eight sequences for two cattle species (*Bos taurus* and *B. indicus*), which acted as hosts of the dipteran flies. The species identification of the dipteran parasites and their hosts was confirmed molecularly and simultaneously by utilising the DNA of

the fly species. The most abundant genera collected were *Musca* and *Chrysomya*, the main decomposer for cattle dung. No significant differences were reported for the fly species composition and species richness between buildings and cage structures. It might be due to the anthropogenic elements of the farm locations as they were near the main public roads, human settlements, and fragmented forests. Even though some of the abundant fly populations (such as the family Tabanidae) did not transmit any pathogens, they were closely associated with the cattle by feeding on dung and played a role as biting livestock pests, leading to stunted growth and reduced milk production. Therefore, this study provides crucial insight into the relationship between parasitic fly species and their Bovidae hosts for future pest management within Malaysia's growing livestock production industry.

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Species Composition and DNA Barcoding of Hemipteran Assemblages Throughout Paddy Growing Seasons

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ABSTRACT

Hemipterans are the diverse, abundant, and important pests in the paddy ecosystem due to their piercing and sucking mouthparts that feed on the crop causing significant losses in rice yields. Despite their important roles in the paddy ecosystem, the information on DNA barcode, diversity, and species richness has been occasionally discussed. This study aimed to measure its abundance, species richness, and barcode hemipteran species from the paddy ecosystem. Active sampling was used with two different sampling arrangements in the paddy ecosystem in Sabak Bernam, Selangor, for two different seasons. Hemipterans were collected and identified up to species level morphologically prior to DNA barcoding. The richness and the abundance of species were measured along with the paddy growth phases (vegetative, reproductive, and mature). A total of 2,167 individuals of seven hemipteran species (*Cyrtorhinus lividipennis*, *Leptocorisa oratorius*, *Nephotettix virescens*, *Cofana spectra*, *Sogatella furcifera*, *Scotinophara coarctata*, and *Graptostethus* sp.) were successfully collected with Shannon-Diversity Index (H' = 0.4572), Margalef richness index (D = 0.7811), and Evenness Index (E = 0.2257). There was no significant difference ($p > 0.05$) for species diversity in both seasons. The highest abundance of hemipteran was during the maturity stage (1,543 individuals), followed by the reproductive (591 individuals) and vegetative stages (33 individuals). This study observed a significant difference

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between the paddy growth for both seasons ($p < 0.05$). Five hemipteran species namely *C. lividipennis*, *L. oratorius*, *N. virescens*, *C. spectra*, and *S. furcifera*, were successfully barcoded with *Leptocorisa*, the dominant genus. Outcomes from this study suggested that different hemipteran management approaches must be developed to cater to different hemipteran species at different paddy growth stages for a successful and sustainable paddy growing practice in Malaysia.

Keywords: *COI*, genetic, hemipteran, Malaysia, pest, rice, true bugs

INTRODUCTION

A paddy field is a habitat for various living organisms such as algae, vertebrates, and invertebrates. The interaction between crops, wild animals and insects result in the increase and decline of crop yield (Saunders et al., 2016). A similar situation could be applied to the insects that play important roles as pests, predators, decomposers, or pollinators in the paddy ecosystem. For example, the data on the abundance and richness of insect species in paddy fields are very important to control the pest populations. In addition, some beneficial insect species will become the natural enemies of pests, which will help reduce pesticides and chemicals in the paddy fields (Ali et al., 2019).

Hemiptera is classified under the combination of two orders, namely Heteroptera and Homoptera, based on molecular evidence of mitochondrial genome sequences (Song et al., 2012). Approximately 50,000 described species of hemipteran have been recorded. However, there is still a lack of information on their diversity in certain ecosystems, such as forest and crop ecosystems, despite their important function in the ecosystem. Furthermore, although hemipteran species have interesting body spots and attractive colouration, some are difficult to identify, especially with very dull body colours and/or similar body structures that can be considered cryptic species (Paterson, 1991). Thus, precise species identification is needed for many purposes, such as in pest control, as a fundamental stage and step in integrated pest management (IPM) (Tahir et al., 2018).

Hemiptera is one of the highest abundances of insects in paddy fields (approximately 30% of the total number of insects) (Sulaiman et al., 2013). This group of insects has significantly reduced paddy production, e.g., the brown planthopper (BPH) and *C. spectra* (Stal) as the dominant pests in Bangladesh and many countries (Rashid et al., 2017), including Malaysia. The BPH and other pest hemipteran species possess sucking mouthpart. Due to that structure, they become the gregarious pest of paddy during vegetative and reproductive stages by sucking nutrients from the plant tissues. Researchers from paddy-producing countries were keen to provide information regarding hemipteran in the paddy ecosystem as this insect group can act as pests or beneficial insects for the

paddy ecosystem. For example, Alves et al. (2016) discussed the spatial distribution and the co-existing pattern of adults and nymphs of the rice stem stink bug, *Tibraca limbativentris*, in paddy cultivations of South America. In Japan, a rapid multiplex polymerase chain reaction (PCR) assay was developed for rapid and precise species identification of several Asian rice planthoppers species (Yashiro & Sanada-Morimura, 2021). The populations of rice grain bug, *Paraeuscosmetus pallicomis*, was also studied in three different paddy ecosystems (weed-free paddy field, weedy paddy field, and paddy dykes) in Indonesia (Abdullah et al., 2017).

Several hemipteran species have been recorded in Malaysia's paddy field: *S. coarctata*, *C. spectra*, *L. oratorius*, *Leptocorisa acuta*, *Cletus punctiger*, *Nezara viridula*, *Lepthocerus indicus*, *Pachybracius pallicornis*, *S. furcifera*, *Recilia dorsalis*, *Nilaparvata lugens*, and *Nephotettix* spp. (Hafizal & Idris, 2013; Hashim et al., 2017; Ooi, 2015; Razali et al., 2015; Sulaiman et al., 2013). Unfortunately, even though information on the species richness was recorded, no information on the barcode and the abundance of the species, especially from Malaysia, is available now.

Numerous diversity studies on insects have been conducted in the Oriental region from conventional and organic paddy fields such as by Ashrith et al. (2017), Meeran et al. (2021), and Zhang et al. (2013). However, related studies that discussed solely hemipteran species composition are still considered lacking and insufficient, suggesting detailed studies need to be conducted to better understand their roles as pests or non-pest in the Malaysian paddy ecosystem. In addition, the barcoding sequences of these species, especially from Malaysia, also provide value-added information for future references. Thus, this study implemented a molecular approach for identifying selected species using DNA barcode analysis to identify an organism using a short DNA sequence (Savolainen et al., 2005). DNA barcode analysis can support morphological identification and give accurate and fast detection results regardless of the insects' life stage (Hebert et al., 2003).

Due to the insufficient data on species composition and genetic data of each species from the paddy ecosystem, this kind of study is highly needed and seems very significant for pest management in the paddy ecosystem. Therefore, this study aimed to measure its abundance and richness as well as barcode the significant hemipteran species in the paddy ecosystem. This study has considered the species from a conventional paddy field in Selangor as a case study site to represent the presence of hemipteran in Peninsular Malaysia from two discontinuous seasons with a different method of insect sampling.

METHODS

Sampling Location

The study was conducted at Parit 4, Sungai Panjang (3° 40'18.1163''N 101° 2'18.9096''E) as a model conventional paddy field management system at Sabak

Bernam, Selangor (middle part of Peninsular Malaysia) at two discontinuously sampling seasons covering three growing phases (vegetation, reproductive, and maturation).

Insect Sampling and Plot Preparation

One hectare, 100 m × 100 m = 10,000 m^2 paddy plot was selected to sample the hemipteran species. The sampling was carried out using active sampling via sweeping nets with two different sampling arrangements for Seasons 1 and 2, regardless of different hemipteran species. For Season 1, sampling was conducted from November 2017 to February 2018. One paddy plot was used as the sampling plot and divided into four subplots with 50 m line transects. Three replications were done at each transect at three different sampling times: 10.00–10.30 a.m., 11.00–11.30 a.m., and 12.00–12.30 p.m. For Season 2, sampling was conducted from November 2019 to February 2020. The sampling plot was conducted on two paddy plots' wards. The sampling plot was a 100 m one-line transect divided into four sections with three replications for each transect at a similar time as in Season 1 (Figure 1).

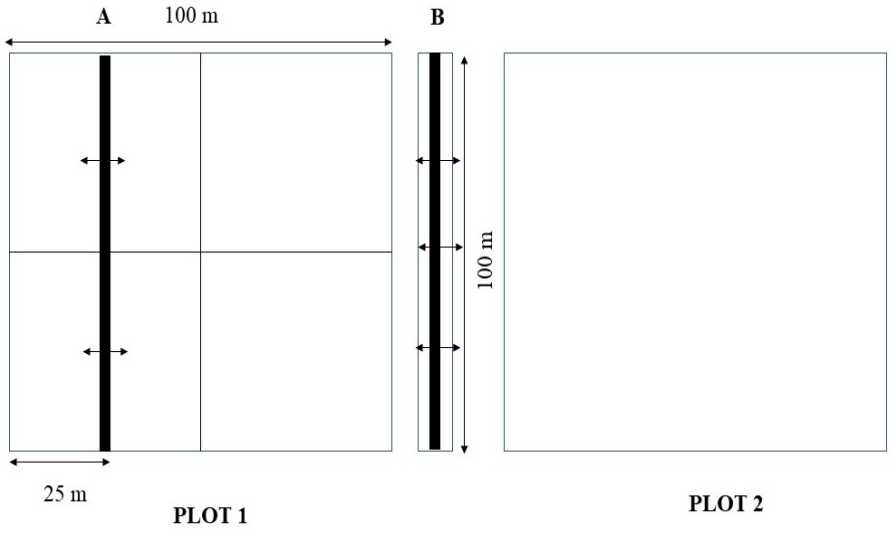

Figure 1. The illustration of sampling plot arrangements, Plot 1 (A) was used for Season 1, and the ward (B) that separated two paddy plots was used for Season 2

Species Identification

Morphological identification of insect species was carried out using Zeiss Stemi DV4 (Germany) stereomicroscope following taxonomic keys by Schaefer (2004), Siwi and Van Doesburg (1984), and Triplehorn et al. (2005). In addition, the identification of insect species was also carried out by comparing the insect collection in the Centre for Insect Systematics (CIS), Universiti

Kebangsaan Malaysia. Insect identification was carried out to the genus and up to species level (if possible).

DNA Barcoding

DNA Extraction and PCR Amplification. DNA was extracted from the insect tissue (of each identified species) using the NucleoSpin DNA kit (Machery-Nagel, Germany) following the manufacturer's protocol regardless of hemipteran species. The PCR for all hemipteran specimens was conducted to amplify the sequence of the extracted DNA using the *cytochrome oxidase* subunit I (*COI*) based on primers designed by Folmer et al. (1994); LCO1490 (5'- GGTCAACA A A T C A T A A A G A T A T T G G - 3 ') and HCO2198 (5'-TAAACTTCAGG GTGACCAAAAAATCA-3') which resulted in 657 bp (excluding forward and reverse primers). The PCR process was carried out in a total of 25 µL reaction comprising of 2 µL DNA template (10–15 ng/µL), 8.50 µL ddH_2O, 0.5 µL 10 mM dNTPs, 2.5 µL PCR buffer 10× (Vivantis, United Kingdom), 1.30 µL 50 mM magnesium chloride ($MgCl_2$), 1.0 µL forward and reverse primers (10 pmol/µL), 0.2 µL *Taq* DNA polymerase (5 U/µL) (Vivantis, United Kingdom) and analysed using MyGene MG96G thermal cycler (China). The PCR condition followed by Halim et al. (2017, 2018) and Shariff et al. (2014); 1 cycle 3 min at 95 °C (denaturation), 40 cycles under 30 s at 95 °C (anneal), 30 s at 47 °C (extension), 1 min at 72 °C for 10 min (final extension). Then, the PCR product underwent electrophoresis for 30 min at 90V using 1.5% agarose gel.

Sequencing and BLAST Analyses. PCR products were sent to Apical Scientific (Malaysia) for sequencing analysis. The DNA sequences were edited and merged using Sequencher™ (version 4.1.4) to combine the forward and reverse sequences. Basic Local Alignment Search Tool (BLAST) software was then used to determine the sequences belonging to the right identified species and referred to the total score, expected value, maximum identical, query coverage, and maximum score (Altschul et al., 1990).

Phylogenetic Analysis. The phylogenetic analysis has been conducted on all the barcoded species to see the species separation at the genus and species level using Phylogenetic Analysis Using Parsimony* and other methods (PAUP*4.0) software (Swofford, 2003). The Neighbour-Joining (NJ) tree was generated by 1,000 replications following the Kimura 2-parameter (K2P) substitution model and bootstrap analysis with 1,000 replications. The NJ tree generated was viewed using FigTree v 1.4.4 (Rambaut, 2009). The most important is the division for the *Leptocorisa* spp. Neighbour-joining (NJ) analysis has been generated using Kimura 2-parameters with 1,000 replications for the bootstrap analysis. The *Thysanoptera* sp. has been selected as an outgroup for the analysis based on the findings obtained by Davis et al. (2010). The genetic distance among species was also conducted under the distance matrix.

Data Analysis. Ecological parameters such as Shannon Diversity Index (*H'*), Evenness Index (*E*), and Margalef Index (*D*) were also carried out based on collected specimens throughout the two seasons. The index's value was obtained using Paleontological Statistic (PAST) software (version 2.17c). The significant difference between paddy growths was measured based on two samples *t*-test by using Minitab v17.1 software.

RESULTS

Composition and Abundance of Species

A total of 2,167 individuals of hemipteran species consisting of seven species *C. lividipennis*, *L. oratorius*, *N. virescens*, *C. spectra*, *S. furcifera*, *S. coarctata*, and *Graptostethus* sp.) under six families (Alydidae, Cicadellidae, Delphacidae, Lygaeidae, Miridae, and Pentatomidae) were successfully collected. *Leptocorisa oratorius* had the highest number with 531 and 1437 from Seasons 1 and 2, respectively (Table 1). The abundance of the hemipteran was the highest during the maturity stage (1,543 individuals), followed by the reproductive (591 individuals) and vegetative stage (33 individuals), respectively.

Table 1

Number of individuals obtained according to family and species from two seasons in the sampling location

Family	Species	No. of individuals during each stage								Grand total
		Season 1				Season 2				
		V	R	M	Total	V	R	M	Total	
Alydidae	*Leptocorisa oratorius* (pest)	0	210	321	531	13	287	1,137	1,437	1,968
Lygaeidae	*Graptostethus* sp. (pest)	0	0	0	0	2	1	3	6	6
Cicadellidae	*Nephotettix virescens* (pest)	0	10	4	14	5	8	14	27	41
	Cofana spectra (pest)	1	11	1	13	0	5	12	17	30
Delphacidae	*Sogatella furcifera* (pest)	0	21	0	21	0	14	21	35	56
Miridae	*Cyrtorhinus lividipennis* (predator)	0	7	0	7	2	3	7	12	19
Pentatomiidae	*Scotinophara coarctata* (pest)	0	0	0	0	10	14	23	47	47
Total number	7	1	259	326	586	32	332	1,217	1,581	2,167

Note. V = Vegetative; R = Reproductive; M = Mature

Species Diversity

Ecological indexes of hemipteran species were calculated along the seasons with the Shannon-Diversity Index (H' = 0.4572), Margalef-richness Index (D = 0.7811), and Evenness Index (E = 0.2257). There was no significant difference ($p = 0.5516$, $p > 0.05$) in the species diversity between the two seasons, while a significant difference was found between growth stages ($p = 0.03768$, $p < 0.05$) for both seasons (Figure 2).

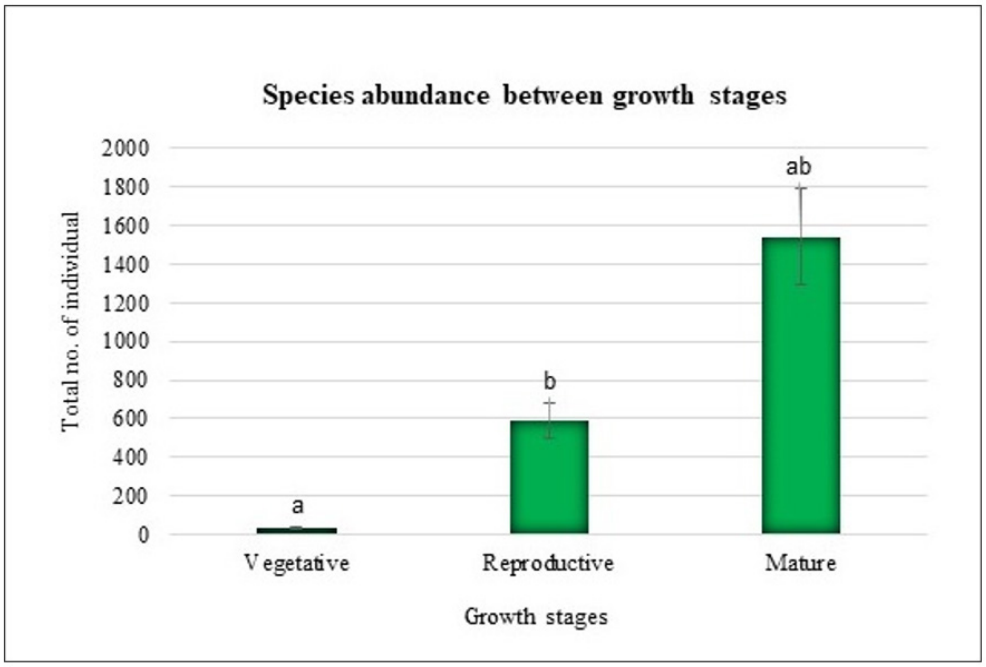

Figure 2. Species diversity between growth stages with different letters on the bar (a, b, and ab) denote the significant differences between growth

DNA Barcode

The morphological identification was made prior to the molecular identification. Several species were successfully identified up to genus level, with limited specimens identified up to species level. Therefore, molecular identification via DNA barcode analysis was carried out on the hemipteran species (Figures 3a-h). During morphological identification, *Leptocorisa* spp. was identified into three morphospecies (Figure 3). However, results from DNA barcoding revealed that all three morphospecies belongs to *L. oratorius* (Table 2). The species separation was presented in the NJ tree supported with bootstrap values to confirm the DNA barcode analysis (Figure 4). The sample for *S. coarctata*, however, failed to amplify. Thus, its DNA barcode is not listed in this study. The genetic distance matrix among species is presented in Table 3.

Table 2

List of species identified based on morphological, molecular identification, and BLAST similarity with the Genbank data (%), total score, expected value, query coverage (%), and maximum score

No.	Species code	Insect species (morphological identification)	Insect species (molecular identification) and GenBank reference	Accession no.	Per cent identity (%) in the BLAST GenBank	Total score	Expected value	Query coverage (%)	Maximum score
1	A1	*Leptocorisa* sp. 1	*Leptocorisa oratorius* (MT277044.1)	OL739348	100	1,133	0.0	85	1,133
2	A2	*Leptocorisa* sp. 2	*Leptocorisa oratorius* (GQ292203.1)	OL739349	99.67	1,096	0.0	83	1,096
3	A3	*Leptocorisa* sp. 3	*Leptocorisa oratorius* (MT277038.1)	OL739347	100	1,133	0.0	83	1,133
4	A4	*Leptocorisa* sp. 4	*Leptocorisa oratorius* (MT277049.1)	OL807628	99.67	1,120	0.0	85	1,120
5	SS1	*Cyrtorhinus lividipennis*	*Cyrtorhinus lividipennis* (KY367198.1)	OL739351	100	944	0.0	71	944
6	SS2	*Leptocorisa* sp. 5	*Leptocorisa oratorius* (MG838383.1)	OL739350	99.61	929	0.0	71	929
7	SS3	*Nephotettix virescens*	*Nephotettix virescens* (OL958679.1)	OL739355	100	1,138	0.0	85	1,138
8	SS4	*Cofana spectra*	*Cofana spectra* (MW577677.1)	OL739353	99.05	1,125	0.0	86	1,125
9	SS5	*Sogatella furcifera*	*Sogatella furcifera* (KC476378.1)	OL739352	99.83	1,090	0.0	82	1,090
10	SS6	*Graptostethus* sp.	*Orius majusculus* (FM210190.1)	OL739354	83.97	549	2e-151	81	549

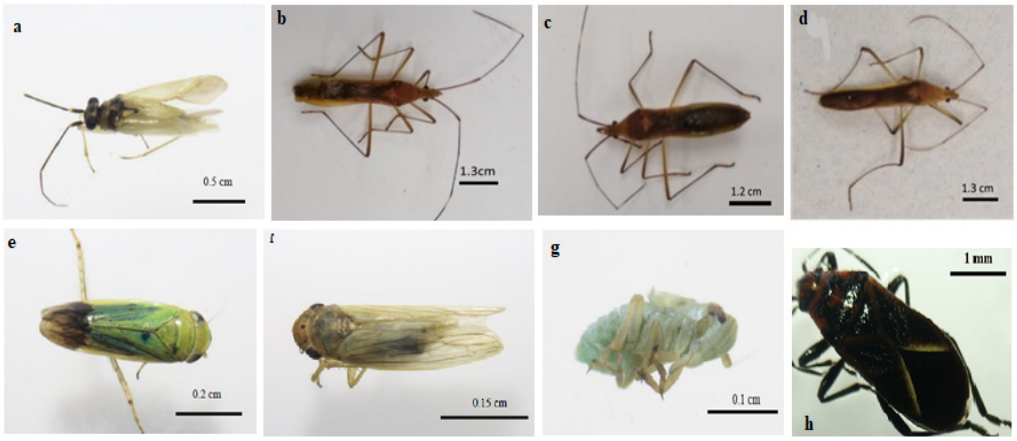

Figure 3. Adults of the hemipteran used for the barcoding analysis and representatives from each species. a. *Cyrtorhinus lividipennis*; b. *Leptocorisa oratorius*; c. *Leptocorisa oratorius*; d. *Leptocorisa oratorius*; e. *Nephotettix virescens;* f. *Cofana spectra*; g. *Sogatella furcifera*; h. *Graptostethus* sp.

Table 3

Genetic distance of the hemipteran species implemented in the phylogenetic analysis in this study

No.	Species	1	2	3	4	5
	A3 *Leptocorisa oratorius*	-				
	A1 *Leptocorisa oratorius*	0.00304	-			
	A2 *Leptocorisa oratorius*	0.00152	0.00152	-		
	A4 *Leptocorisa oratorius*	0.00152	0.00457	0.00304	-	
	SS2 *Leptocorisa oratorius*	0.00152	0.00457	0.00304	0.00000	-
	Cyrtorhinus lividipennis	0.20852	0.20700	0.20852	0.20700	0.20700
	Sogatella furcifera	0.27245	0.27093	0.27245	0.27093	0.27093
	Cofana spectra	0.22222	0.22070	0.22222	0.22070	0.22070
	Orius majusculus	0.22222	0.22070	0.22222	0.22374	0.22374
	Nephotettix virescens	0.23896	0.23592	0.23744	0.23744	0.23744
	HQ986473.1 *Thysanoptera* sp.	0.60397	0.60092	0.60244	0.60398	0.60398

No.	Species	6	7	8	9	10	11
	A3 *Leptocorisa oratorius*						
	A1 *Leptocorisa oratorius*						
	A2 *Leptocorisa oratorius*						
	A4 *Leptocorisa oratorius*						
	SS2 *Leptocorisa oratorius*						
	Cyrtorhinus lividipennis	-					
	Sogatella furcifera	0.27549	-				
	Cofana spectra	0.23135	0.27245	-			
	Orius majusculus	0.21309	0.27702	0.22679	-		
	Nephotettix virescens	0.23440	0.29072	0.19482	0.22070	-	
	HQ986473.1 *Thysanoptera* sp.	0.60986	0.60374	0.59318	0.59003	0.59641	-

Based on the neighbour-joining tree, the separation of each species is presented (Figure 4). First, *Thysanoptera* sp. was chosen as the outgroup and located at the most basal of the tree. Next, the *S. furcifera* was situated at the basal of the ingroup tree, followed by a clade consisting of *N. virescens* and *C. spectra* with a bootstrap value of 92. Next, *C. lividipennis* and *Graptostethus* sp. were formed in the inner clade before separating into one big clade consisting of five individuals of *L. oratorius* with a bootstrap value of 100.

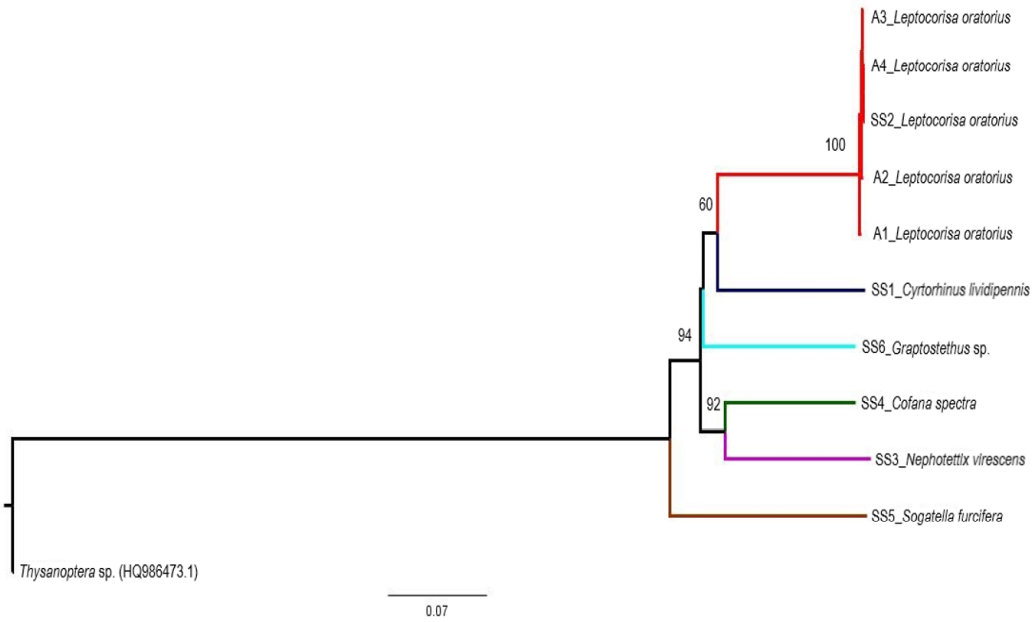

Figure 4. NJ tree of the hemipteran species that was implemented in the barcoding analysis. The number of bootstrap values is on the branches

DISCUSSION

This study presents the barcoding information for the hemipteran species collected from the paddy ecosystem. In addition, the richness and the abundance of the hemipteran species assemblages were also studied from the model sampling site of the conventional practice in Peninsular Malaysia with different sampling arrangements. To date, there is no study published solely on the hemipteran species from the paddy ecosystem in Malaysia and the neighbouring countries. However, several published studies from Malaysia and other Asian countries have focussed on the general diversity of the insects, including Hemiptera as pest, predator, pollinator, and decomposer with specific ecological functions (Nasiruddin & Roy, 2012; Rashid et al., 2017; Razali et al., 2015; Sulaiman et al., 2013).

The identification of five insect species (*C. spectra*, *N. virescens*, *L. oratorius*, *S. furcifera*, and *C. lividipennis*) was carried out using DNA barcode analysis. Most of the sequences available in the Genbank, however, are not from Malaysia. Therefore, our study contributed to the new sequences for the Malaysian species. According to Hebert and Gregory (2005), DNA barcode analysis provides easy access for the public to identify scientific names and biological properties of any species on the planet. In addition, DNA barcoding can accelerate the discovery of new species through rapid species identification and divergence of taxa that may represent new species. Interestingly, data obtained from this study is very important for precisely identifying species, especially the pest species, through the barcode information, as stated in Yaakop et al. (2020).

Except for *S. coarctata*, all the barcodes of hemipteran species in this study are available in the Genbank database. BLAST results also showed that a sample (SS6) could not be identified due to the low percentage similarity (*Orius majusculus* = 84%). The similarity percentage of more than 97% can be applied for identification of up to species level, while the value of more than 95% only applied to genus level (Rosenthal et al., 2017). Hence, this sample was identified based on morphological characteristics, resulting in *Graptostethus* sp. (Lygaeidae).

In this study, the DNA barcodes of five individuals of *L. oratorius* were obtained (Table 2). All the individuals were difficult to be identified morphologically due to high similarities based on the spot, the body marking, and the colouration (Siwi & van Doesburg, 1984), which always leads to misidentification. The samples collected were confused with *L. acuta*, *Leptocorisa varicornis*, and *Leptocorisa chinensis*, which are also pest species for paddy fields (Cobblah & Den Hollander, 1992; Ishizaki et al., 2007; Rattanapun, 2013). All four species of *Leptocorisa* is known as rice seed bug and have similar biology, as adults and nymphs suck out the rice grain, causing heavy damage and even emptying the rice seed (Dong et al., 2021). Besides, all these four species also share the same habitat and host plant, which are grasses and flowering rice (Torres et al., 2010). However, in this study, only *L. oratorius* was identified from the sampling area.

DNA barcoding data helps identify the smaller insects (leafhoppers) and insects that are difficult to distinguish based on morphological characteristics, especially after preservation in ethanol, such as white-backed planthopper *S. furcifera* and white leafhopper *C. spectra*. *Cofana spectra* is considered the minor pest for paddy, while the *S. furcifera* is an important pest known as a phloem feeder. The species was easily identified based on the body colouration and might transmit the Tungo virus into the paddy plant (Dasgupta et al., 1991).

Cyrtorhinus lividipennis has larger body sizes (2–5 mm) and acts as generalist predators for planthoppers and leafhoppers in the paddy field (Cohen et al., 1994; Reissig et al., 1986). The sequence for this

species is newly deposited into the Genbank. However, the DNA barcode analysis for the *S. coarctata* (Pentatomidae) was not carried out due to difficulty obtaining the DNA sequence. However, the effort to obtain the barcode information for the near future of the species is crucial.

This study's neighbour-joining (NJ) tree supported the molecular identification species apart from the BLAST result. The separation of each species with high bootstrap values provided strong support for each morphological and molecular identification. In the NJ tree, all the species were separated, while *L. oratorius* was confirmed as one species as all five individuals formed in one clade with 100 bootstrap supports.

Abundance and Species Richness

A total of six families with seven genera of pests and beneficial insects were successfully collected from this study. The hemipteran species richness, which refers to the pests in this study, was considered high compared to the previous studies in the paddy field. For example, Sulaiman et al. (2013) recorded only five species of potential pests with three uncommon species, namely *N. viridula*, *Riptortus linearis*, and *P. pallicornis*. Razali et al. (2015) conducted a diversity study in Tanjong Karang, Selangor, and recorded the presence of *L. oratorius* and *C. spectra* in the sampling sites. A study of insect diversity in Bangladesh by Nasiruddin and Roy (2012) also sampled similar insect pests such as *N. virescens*, *S. furcifera*, and *C. spectra*.

The hemipteran insects obtained were commonly found in the Asian paddy fields. There were two main insect groups: insect pests and beneficial insects. By referring to International Rice Research Institute (IRRI) (2022), the main paddy insect pests found in this study were *L. oratorius*, *N. virescens*, *C. spectra*, and *S. furcifera*. A predator insect species found in this study was *C. lividipennis* (Chandrasekar et al., 2017). A potential pest (*Graptostethus* sp.: Lygaeidae) was also reported in this study. According to Malipatil et al. (2020), a well-known species of *Graptostethus* (*Graptostethus servus*) is a granivore hemipteran and an irregular pest of pulses in Australia. This species also reported injuring cotton, sunflower, beans, nuts, and several ornamental plants outside Australia (Sweet, 2000). Further investigation needs to be conducted to verify the role of *Graptostethus* sp. in this study to avoid the surge of a new potential pest for paddy cultivations in Malaysia.

The results showed that insect species' diversity varied at three stages of paddy growth at two different discontinuous seasons. According to Bambaradeniya et al. (2004), flora and fauna recorded in the paddy fields vary according to the cycle of paddy cultivation because each paddy phenology phase growth has different uses for fertilizers, water levels, and insecticides. Based on these two seasons, the sampling was conducted during the same period each year; therefore, the climatic condition was considered a similar condition. However, the result showed non-significance differences in abundance and richness, even though

the different arrangements of collecting hemipterans were implemented (Figure 1).

In this study, the species abundance was the highest during the maturation stage, followed by the reproduction, and the vegetation stage showed the lowest number in both seasons (Table 1). The analyses showed the non-significance difference between growth stages in species diversity because, during the maturation stage, the hemipteran pests were actively infesting the plant by sucking the paddy stems; thus, the number of species was dominated by the *Leptocorisa* species in this study. Referring to Su et al. (2015), during the maturation stage, the hemipteran species have enough food sources, and *Leptocorisa* spp. will start to damage the paddy plant by sucking the paddy milk, resulting in the empty or half fill of grains (Kim et al., 2017).

According to Reissig et al. (1986), *L. chinensis* and *L. oratorius* infest the paddy plant maturely because their nymph and adult will suck paddy stem fluid and endosperms. In addition, based on the observations made during the sampling, there was also the presence of weeds along the path between paddy fields. The weeds could contribute to the abundance of rice bugs as they provided another habitat and reservoir for rice bugs apart from paddy fields. This statement is supported by the study of Kainoh et al. (1980), who clarified the presence of rice bugs along with the weeds in between passage of the paddy fields. Pathak and Khan (1994) also found that the abundance of rice bugs in paddy fields was contributed by the presence of forest areas, weeds, and paddy cultivation in several stages. Adults rice bug uses weeds and other nearby host plants as their habitat and food sources before moving to paddy during the reproductive stage.

Despite the samplings being done at two discontinuous seasons with two different sampling arrangements, *t*-test analysis showed no significant difference in the species abundance for the total and individual species. The analysis showed that different arrangements of insect sampling at Seasons 1 and 2 showed no significant difference in species abundance for the total and individual species. The results revealed that the different arrangements in samplings would not affect the species abundance and diversity. According to Rothschild (1970), *Leptocorisa* species have active movement and have been proven based on their mark-release recapture technique at the paddy ecosystem in Sarawak. Thus, we estimated that the number of *Leptocorisa* spp. might be much higher in the real situation.

In addition, the different paddy management systems are also considered one of the major causes of the differences in insect abundance. The conventional paddy management systems integrate insecticide and kill the non-targeted insects, such as natural enemies, which control the insect pest populations in paddy fields (Namara et al., 2013). According to the information obtained from the field manager of the sampling location, pesticides will be applied upon symptoms and the presence of pests in the paddy fields. Therefore, the frequent use of insecticides will affect beneficial

insect populations as well as increase the pest populations indirectly. The results of a study by Cohen et al. (1994) revealed that the effect of insecticides in paddy fields was dissimilar according to different pests and caused dynamically populated insects.

CONCLUSION

The diversity and the DNA barcodes of the hemipteran species in the study area are presented. Based on the findings, the abundance and the species richness were also affected by the method of sampling and insects' behaviour, hence suggesting several effective sampling methods such as using baited traps to estimate the diversity of the species group accurately. This study can be used as a guideline for decision-making strategies to implement different hemipteran management approaches to combat different hemipteran pest species for each paddy growth stage for a successful hemipteran pest management strategy. Farmers, managers, and stakeholders are obliged to monitor and prevent the aggressive growth of the wild weeds nearby their paddy plots that contribute to the food sources and the breeding sites for the hemipteran pest species. Furthermore, the high abundance of pest species also contributes to the increase of its natural enemies. Therefore, the study of the hemipteran pests, natural enemies (parasitoids and predators) and their DNA barcoding information is very important prior to apply the biological control agents, which are very promising for zero use of insecticides in the paddy ecosystem.

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Alternate Wetting and Drying (AWD) on Rice Irrigation

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ABSTRACT

In Malaysia, numerous methods have been subsequently established subjected to water-saving irrigation aiming to improve the common conventional irrigation system. However, among the most preferred water-saving method, alternate wetting and drying (AWD) irrigation adoption are presently in paddy cultivation yet has very limited information, especially locally. Hence, this study intended to propose two treatments, namely continuously flooded (control) and AWD irrigation, to investigate the feasibility of AWD implementation. The experiment was conducted at the paddy field of Padang Raja Kelantan, Malaysia. From the result, the agronomic performance was evaluated by several attributes under the growth performance evaluation, grain yield performance evaluation, and chlorophyll measurement. Statistical analysis was performed on the obtained data, and both growth, yield performances, and chlorophyll content resulted in no significant difference at $p < 0.05$, a 95% confidence level.

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INTRODUCTION

The water crisis is a major concern in Malaysia as the water demand in growing areas is gradually increasing. Despite having an average rainfall of 3,000 mm, considered

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among the highest rainfall intensity in other Asian countries, the main water sources can no longer meet the increasing demand for the domestic, industrial, and agricultural sectors. In agricultural areas, water is a prime factor and important resource needed for proper crop growth, particularly for water-intensive crops such as paddy. Malaysia has a long and strong history of paddy cultivation. Farmers usually adopt conventional practices where paddy is grown under continuously flooded conditions. This traditional practice commonly requires standing water per season, ranging from 700 mm to 1,500 mm (Oliver et al., 2010). Nonetheless, this practice has a long-term issue concerning the environmental effect of unnecessary irrigation water consumption.

Given water conservation, the most practical approach is water usage optimization. Numerous water-saving techniques have been introduced and documented since time immemorial, for example, intermittent irrigation, drip irrigation, deficit water regime, a system of rice intensification (SRI), and alternate wetting and drying (AWD). AWD is the most popular water-saving technology adopted to improve water use efficiency. In the 90s, International Rice Research Institute (IRRI) introduced a practical and advanced technology approach focusing on water-saving management practice, known as the 'alternate wetting and drying (AWD) technique'. The enforcement of AWD is farmer-friendly.

Water conservation technology only needs a proper field water tube made from a low-cost material, such as bamboo and polyvinyl chloride (PVC). A field water tube is used to monitor the standing water level. The paddy field is flooded with irrigation water and is allowed to dry out to a certain ground depth before the irrigation water is reapplied again. In AWD practices, less irrigation water input is required. Past researchers (Akter et al., 2018; Aziz et al., 2018; Dong et al., 2018; Zhuang et al., 2019) have reported and acknowledged this technique and found that by using AWD practice, there is no significant decrease in yield compared to continuous flooded practice. In AWD irrigation, not only does a reduction of up to 30% of total irrigation water input, but the total water productivity is also increased, and the same goes for the nutrient uptake (Sarkar, 2001).

AWD irrigation has been widely used worldwide and is one of the popular methods in paddy cultivation. AWD has promoted water productivity in rice irrigation relative to conventional irrigation (Akter et al., 2018; Biswas et al., 2021; Busari et al., 2019; Chidthaisong et al., 2018). In addition, Norton et al. (2017) ascertained in their report that AWD increased the total grain mass due to the high number of productive tillers. It was supported by Carrijo et al. (2018) and Sriphirom et al. (2018), who mentioned that the AWD practice positively affects the tiller, panicle numbers, and grain yield.

However, in Malaysia, the farmers' adoption of AWD is still small. It may be due to a lack of information, awareness, expertise, and successful experimental

evidence. Based on the previous related research, the study on AWD in paddy growth and grain yield performances has been under research, especially locally. Thus, this study aimed to investigate the feasibility of AWD adoption subjected to agronomic physiology performances in Kelantan, Malaysia.

MATERIALS AND METHODS

Description of the Study Area

Kelantan district experienced a tropical climate divided into the wet and dry seasons. The highest rainfall precipitation can be recorded from November to January, while the highest temperature is observed from February to October (Figure 1). This study was conducted at the end plot of rice cultivation area at the paddy field of the Department of Agriculture Kelantan Research and Developmental Platform Padang Raja Kelantan, Kelantan Malaysia (5°57'44.4"N 102°17'20.3"E). The area is mostly covered with alluvium deposits consisting of unconsolidated to semi-consolidated gravel, sand, clay, and silts. The underlain layer is mainly dominated by granitic and sedimentary/metasedimentary rocks that consist of sandstone, phyllite, slate, and shale (Ilahi et al., 2021).

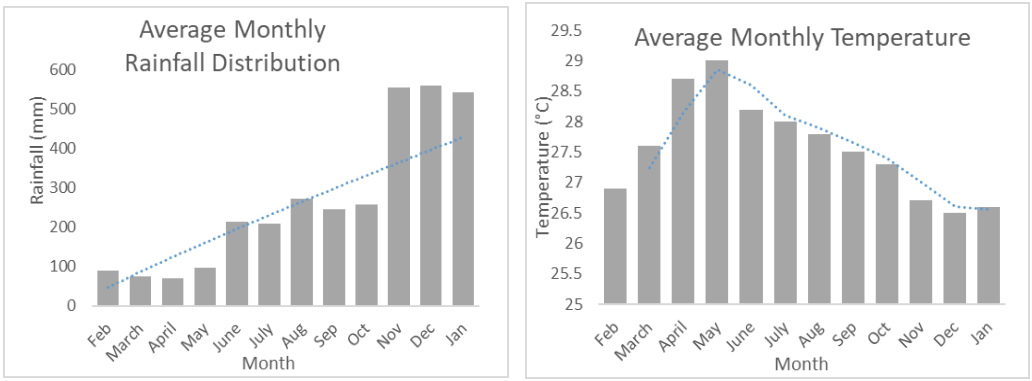


Figure 1. Average monthly rainfall distribution and temperature recorded from the Malaysian Meteorological Department in Kelantan, Malaysia

Experimental Design

Two main water management studies: continuously flooded condition (control) and AWD irrigation, were proposed. The experiment was initiated in 2020 and arranged as a randomized complete block design (RCBD) plot with three replications. Treatment plots were designed to be 46 m x 50 m and separated with compacted earth bunds to minimize and block lateral water seepage between plots. Drainage for outflow water was properly constructed to avoid excessive standing water remaining in the field plot, especially in heavy rainfall. Paddy rice (*Oryza sativa* L.) variety of Mardi Sempadan MR303 was chosen and grown during the wet season (June to September). The direct seeded method was applied for both water management practices. Nutrient management was not a focus in

this experiment; thus, the fertilizers and pesticides used for all plots during the whole season of rice cultivation were standardized based on the local farmer's utilization.

A control experiment was proposed by adopting practices from the normal conventional systems by local farmers. The treatment plot was regularly irrigated, keeping 5 cm to 10 cm of standing water starting from 1 day after sowing (DAS). From 85 DAS onwards, plots were not irrigated before harvesting (120 DAS), and soil was allowed to dry out. Following the recommendation by IRRI, the safe-AWD experiment was proposed, in which a low-cost field water tube built from polyvinyl chloride (PVC) pipe was used to monitor the water level at a depth of 15 cm below the ground surface. The field water tube was 15 cm in diameter with 30 cm in height; half of the size was perforated with holes to ensure water could flow in and out of the tube. The 15 cm non-perforated part of the tube protruded above the ground surface, while another half was hammered into the field. The soil inside the tube was removed until the bottom of it could be visibly seen. The plot was regularly irrigated by letting the standing water fluctuate between 5 cm to 10 cm till 30 DAS. It ensures that the paddy crop establishes a stable physiological state before the experiment starts. Afterward, the AWD experiment began by allowing the water level to drop to 15 cm below the ground surface; after that, the irrigation was re-applied till the standing water reached 5 cm above the ground surface. After that, the AWD irrigation was reapplied again till the harvest stage. The AWD experiment started at 30 DAS to suppress weeds from growing and stabilizing the seed from water stress.

Sampling Parameter

Table 1 summarizes the overall sampling parameter and evaluation of samples for both growth and yield performance of paddy crops for two water management (control and AWD irrigation). Daily observations were made for standing water depth in a control experiment to keep it fluctuating from 5 cm to 10 cm. In the AWD experiment, the dates of standing water disappeared, and re-flooded times were recorded. In addition, quantitative measurement was recorded at each growth stage: plant height, tiller number, and average soil plant analysis development (SPAD) measurement between three healthy leaves. SPAD is a tool measuring the chlorophyll content indirectly. SPAD values were measured thrice in each treatment plot, and the average value was recorded using SPAD-502 Plus (Konica Minolta Sensing, Japan).

Evaluation of crop growth performance was evaluated from samples collected at the individual plot of 0.5 m^2 area and at the center of an experimental plot to avoid the border effect. Destructive analysis was conducted for better evaluation of paddy crop samples. Plant samples were separated for grain, leaf, and stem. The plant material afterward was dried till to constant weight.

Grain yield performance was evaluated from five panicles collected randomly. The panicle length, the total number of grains, and the number of filled and unfilled grains

were measured and counted. In addition, all grains samples were dried to constant weight, and the dry weight of filled and unfilled grains, the weight of 1,000 grains, percentage grain filing, and harvest index was calculated. The equation involved is shown below:

$$\text{Harvest index (Karki et al., 2018)} = \frac{\text{Economic yield (grain yield)}}{\text{Biological yield (grain yield + straw yield)}} \quad (1)$$

Table 1

List of sampling parameters and evaluation of samples observation for both growth and yield performance of paddy crop for two water management (control and AWD irrigation)

Observation	
Daily observation	
i.	Standing water disappearance
ii.	Re-flooding time
At each growth stage	
i.	Plant height
ii.	Tiller number
iii.	SPAD measurement
Evaluation of crop growth	
i.	Samples were collected at the individual plot of 0.5 m^2
ii.	Samples were separated for grain, leaf, and stem
iii.	Samples were marked
iv.	Samples were dried to constant weight
Evaluation of grain yield	
i.	Samples were collected from five panicles at random
ii.	Samples were measured for their panicle length, the total number of grains, number of filled and unfilled grains
iii.	Samples were dried to constant weight
iv.	The weight of 1000 grains, % of grain filling, and harvest index were calculated

Statistical Analysis

Three replicates were used for each treatment, and the effects of treatments on paddy crop growth and grain yield performance were statistically analyzed by *t*-test using Excel 2011 (Microsoft Corporation, USA). *P*-value was < 0.05, and the differences were considered significant.

RESULTS AND DISCUSSION

Various research and yet ongoing methods were predetermined to improve the current practice of irrigation systems to reduce irrigation water consumption ultimately. The concern of introducing the AWD water management in this study is to investigate the potential effect of the AWD on agronomic

traits of crop growth performance, grain yield performance, and chlorophyll content measurement. The crop growth performance for both water management practices was determined by plant height, panicle length, no of filled and unfilled grains, while the grain yield performance was determined by total grain number per panicle, grain filling (%), 1,000-grain weight, and harvest index (Thakur et al., 2018).

Table 2 represents the growth performance between two water management treatments: control and AWD irrigation technique, and was illustrated in the graph as in Figure 2. All values were presented as mean ± standard error (SE) (n = 3) of three replicates at $p < 0.05$ and 95% confidence level. Table 3 represented the grain yield performance between two water management treatments: control and AWD irrigation technique, and was illustrated in the graph as in Figure 3. All values are presented as mean ± standard error (SE) (n = 3) of three replicates at $p < 0.05$ and 95% confidence level. Figure 4 visualizes water level measurement for water management (control and AWD irrigation) throughout the growing season.

Table 2

Growth performance of paddy crop for two water management (control and AWD irrigation). Values are mean ± SE (n = 3) of three replicates at p < *0.05*

Treatment	Plant height (cm)	Panicle length (cm)	No. of filled grains	No. of unfilled grains
Control	99.78 ± 4.15	24.17 ± 0.55	121.93 ± 8.80	14.00 ± 2.96
AWD	95.00 ± 4.53	22.31 ± 0.58	94.40 ± 5.35	10.93 ± 2.10
Significance	ns	ns	ns	ns

Note. ns = Not significant

Table 3

Grain yield performance and harvest index of plant crop at harvest stage for control and AWD irrigation. Values are mean ± SE (n = 3) of three replicates at p < *0.05*

Treatment	Total grain number/panicle	Grain filling (%)	1,000-grain weight (g)	Harvest index
Control	135.93 ± 6.17	89.48 ± 2.65	71.15 ± 4.51	0.46 ± 0.03
AWD	105.33 ± 4.90	89.58 ± 2.09	83.42 ± 7.83	0.47 ± 0.03
Significance	ns	ns	ns	ns

Note. ns = Not significant

Based on Tables 2 and 3, the growth performances and grain yield performances result in no significance at *p*-value < alpha value 0.05 subjected to control and AWD treatment, and this was supported by previous studies of Carrijo et al. (2017), Ishfaq et al. (2020), and Yao et al. (2012). Studies show that the reasoning behind the insignificant difference between both treatments was due to no water stress taking place in AWD irrigation as Safe-AWD was utilized. In Safe-AWD, the standing water was allowed to drop until 15 cm below the ground surface, and irrigation was re-applied afterward, keeping it to 5 cm above the ground surface. It ensured the soil water potential was at > -20kPa, indicating the limitation to mild water stress. Moreover, the experiment was conducted during the wet season, when the precipitation rate exceeded the average. It was in agreement with previous research reported by Chidthaisong et al. (2018) and Sriphirom et al. (2019) that specifically mentioned that the implementation of AWD during the monsoon season was rather hard due to the difficulty of managing the level of standing water. Setyanto et al. (2018) further mentioned that if AWD were properly implemented, it would enhance the root growth and eventually increase the grain filling rate by improving the mobilization of reserve carbon to grains.

Figure 5 shows the image of the paddy crop between control and AWD irrigation water management on the growing days at 60 DAS, 90 DAS, and 115 DAS (before harvest time). At the control treatment, the growth performance comprised plant height, panicle length, and numbers of filled and unfilled grains showed higher value than in the irrigation method. On early 60 DAS of paddy crop (Figure 6), the plant height was higher in control than AWD water management, and the results in Table 2 supported this.

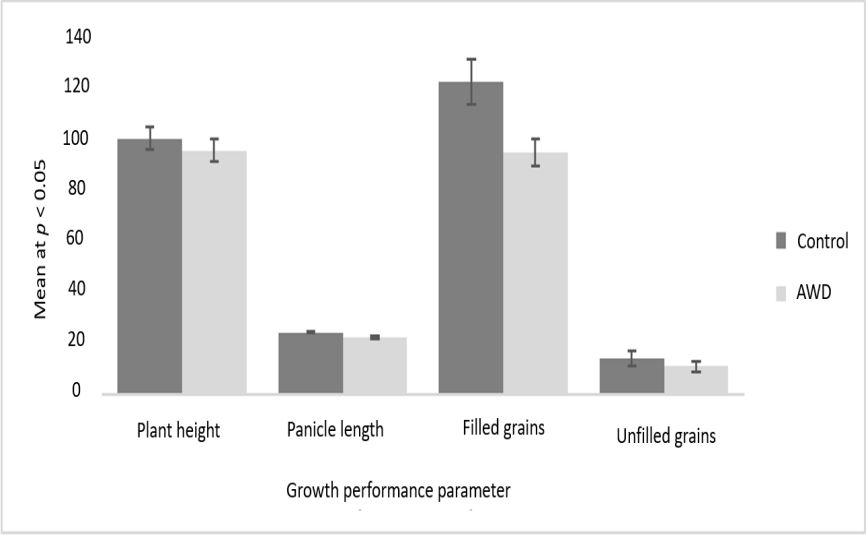
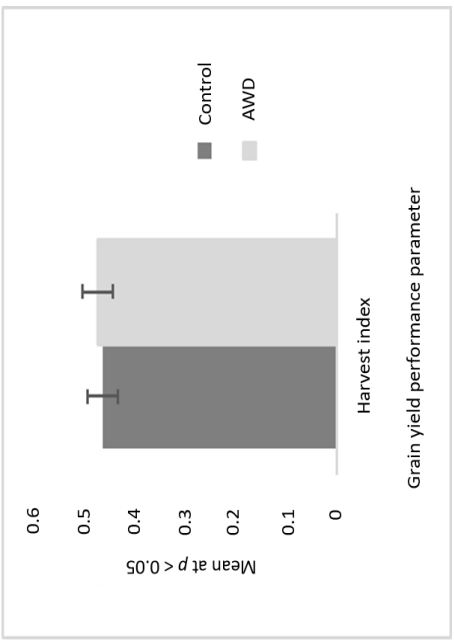


Figure 2. Paddy crop growth performance parameter for two water management (control and AWD irrigation)



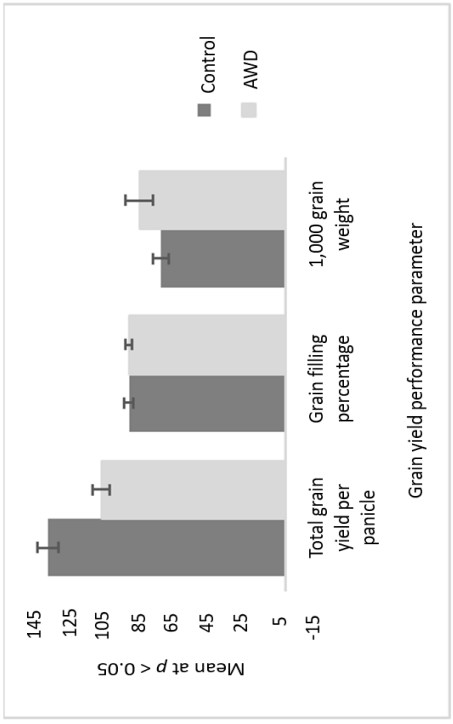
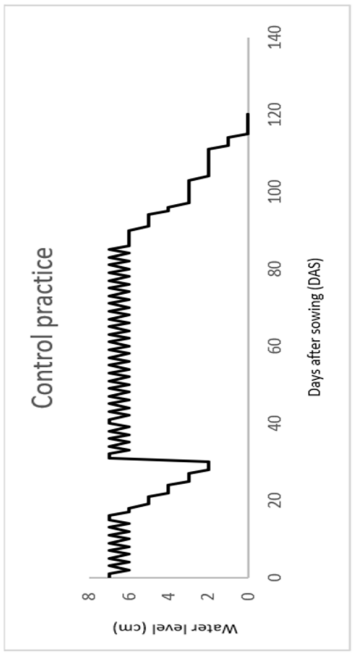


Figure 3. Grain yield performance parameter for two water management (control and AWD irrigation)



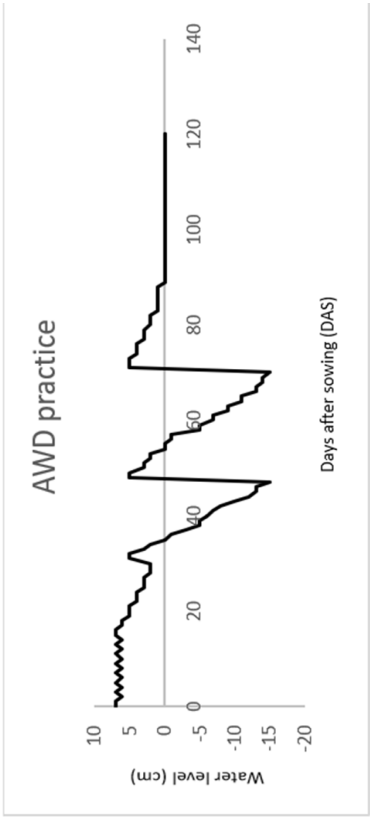


Figure 4. Water level management for two water management (control and AWD irrigation)

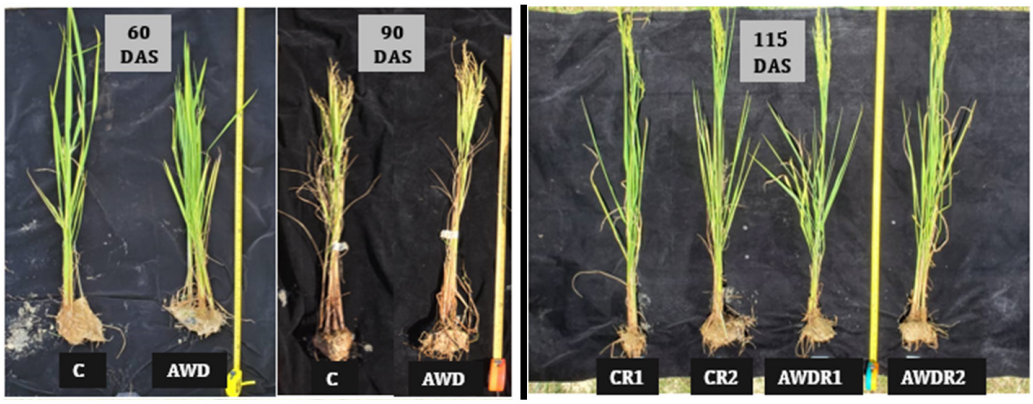


Figure 5. Paddy crop growth comparison between control and AWD irrigation water management at 60 DAS, 90 DAS, and 115 DAS

Nonetheless, on 90 DAS, the plant height was taller in AWD compared to control in a slight difference. As harvest time approached, the plant height was comparative in both treatments. The lower height in AWD irrigation agreed with past studies by Busari et al. (2019) and Fonteh et al. (2013), which have proven that irrigation water management in AWD has significantly reduced the plant height. The plant height and panicle length significantly influence the filled and unfilled number. As plant height increases, so thus the panicle length increases, and the total number of grains increases. Pascual et al. (2017) have mentioned that the number of unfilled grains may increase as water stress increases, thus affecting the grain filling process. It may result to yield reduction as spikelet sterility has been disrupted.

However, at grain yield performances, the percentage of grain filling, 1,000-grain weight, and harvest index show higher value in AWD compared to control. In contrast, the total number of grains per panicle was higher in control than in AWD (Table 3 and Figure 5). AWD method has been proven to have a greater influence on increasing the grain yield (Liu et al., 2013); however, the importance behind the practice was to control the threshold level of soil water potential. As practiced by Ishfaq et al. (2020) and Zhang et al. (2021), results from the safe AWD regime show that re-applied irrigation water at 15 cm to 20 cm of soil depth while maintaining the soil water potential at -20 kPa can avoid paddy crops from experiencing severe water stress. It was supported by Norton et al. (2017), which mentioned the importance of AWD adoption on the drying cycle's duration.

The AWD regime was also believed to increase the percentage of grain filing and 1,000-grain weight, thus increasing the percentage of reproductive tillers and dry matter (Liu et al., 2013). However, this was opposite to the report by Pascual et al. (2017) that mentioned that water stress might affect the reproductive tillers' number and thus may disturb the panicle initiation process.

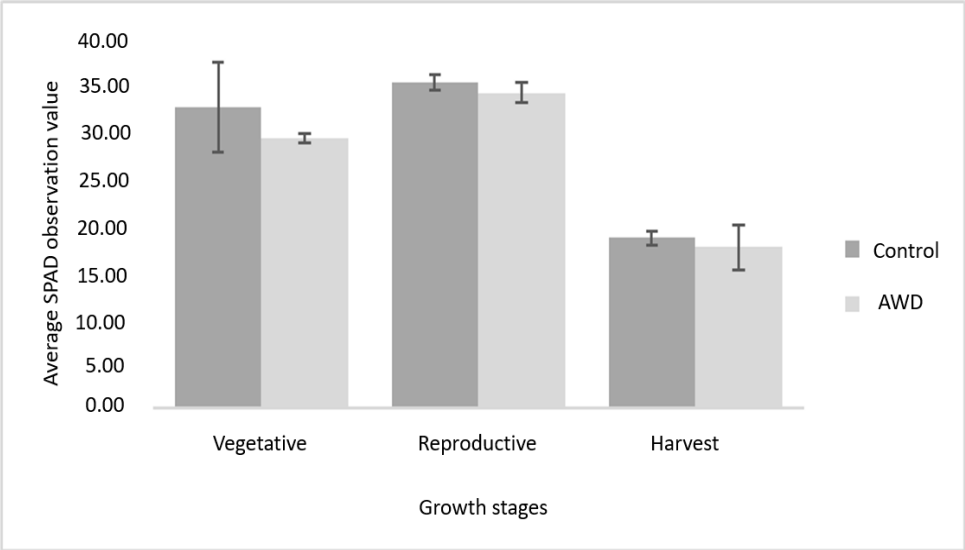


Figure 6. Average SPAD measurement of paddy crop at the vegetative stage, reproductive stage, and harvest stage for control and AWD irrigation

Figure 6 indicates the average chlorophyll content reading measured using the SPAD meter during the vegetative, reproductive, and harvest stages for control and AWD irrigation. SPAD reading observation was measured at three random leaves for both treatments, as represented in Figure 5. Among the vegetative, reproductive, and harvest stage, the average SPAD value was higher in the vegetative stage compared to AWD in all replications. SPAD was used widely to measure chlorophyll content in crop growth stages indirectly. The previous study by Asa et al. (2011) reported that the range difference in SPAD readings was dependent on changes in paddy growth maturity. The higher SPAD value indicates active photosynthesis activities occurred. It was relatable at the early vegetative stage when the crop developed leaves and tillers and increased plant height. While at the reproductive stage, the initial panicle has started to emerge from the stem, and at this stage, the SPAD measurements were recorded consistently at a high value. It indicates that the process of photosynthesis was constantly happening.

CONCLUSION

The irrigation consumption was relatively higher in control than in the AWD technique, as the standing water requirement throughout the season for control was within the range of 5 cm to 10 cm. However, both treatments showed no significance in crop growth performances. However, the AWD irrigation method was preferable in water-saving since the irrigation was scheduled to alternately wet and dry the paddy crop field; thus, the irrigation water consumption is much less than the continuously flooded irrigation. Therefore, Yao et al. (2017) supported the

AWD method as the preferable method, resulting in high grain yield and high-water efficiency.

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Shorea macrophylla: Overview of Illipe Nut Producing Tree

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ABSTRACT

Shorea macrophylla is also named as 'Engkabang' (Iban), 'Kawang' (Brunei), and 'Tengkawang' (Indonesia). It belongs to the Dipterocarpaceae family and is categorised under the genus *Shorea*, which can be found in the tropical rainforests in Southeast Asia. It prefers wet habitats, such as in periodically flooded alluvium and riverbanks. The tree size of *S. macrophylla* is medium or large; however, some researchers in Kalimantan claim it is a small tree. The flowering and fruiting systems of most Dipterocarps, including *S. macrophylla*, are irregular, but there is a massive flowering event once every few years. Its genetic structure is characterised by moderate genetic diversity within species and populations, as well as high genetic differentiation within local populations. *Shorea macrophylla*, also known as the Light Red Meranti, is a suitable timber supply for light construction work. It produces illipe nuts that are widely used as cocoa butter replacer fat. Wildlife eats the ripe illipe nuts, which contain a high oil content with mostly beneficial unsaturated triglycerides. The fat extracted from the nut are suitable for cosmetic application as it provides a good moisturising effect. Future investigation into the illipe nuts' composition and other potential uses should be carried out.

Keywords: Dipterocarpaceae, Engkabang, illipe nut, *Shorea macrophylla*, timber

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INTRODUCTION

Shorea macrophylla, also known as Engkabang tree by the locals, are grouped under the Dipterocarpaceae family. Dipterocarpaceae, including *S. macrophylla*, is mainly distributed in Southeast Asia, and they favoured the periodically flooded alluvium and grew wild by the riverbank. Its fruit, the 'illipe nut', is rich in oil content,

and thus *S. macrophylla* is also named the 'butter in the forest' by the locals. The illipe nut is one of the foods consumed by wild animals such as fish. Interestingly, *Tor tambroides* preferred high protein-energy content, including Engkabang fruit, which could be the main contributor to its sweetness-enriched flesh (Rahman & Basri, 2013). However, the flowering and fruiting of all the dipterocarps, including *S. macrophylla*, is irregular (Mathiesen, 1994). There is a mass flowering occurrence once every few years, and the locals would collect the illipe nuts and dry it for a prolonged storage purpose for other uses later (Chai, 1998). According to Chai (1998) as well as Sulistyawati and Widyatmoko (2018), *Shorea gysbertsiana* Burck, a species from the same genus, is another name for *Shorea macrophylla*. However, Nurtjahjaningsih et al. (2012) found that the allele frequencies of these two species are different, indicating that they are closely related but not the same.

DISTRIBUTION OF *SHOREA* SPP.

The tropical rainforests of Borneo, Malaysia, Indonesia, and the Philippines are abundant in *Shorea macrophylla* and other Dipterocarp species (Chai, 1998; Ng et al., 2002). It prefers the wet habitats, which can be found in periodically flooded alluvium and along some riverbanks up to 1,500 metres above sea level, but with rare and scattered distributions across hillsides below 600 metres (Kanzaki et al., 1996; Lee et al., 1997; Ng et al., 2002). *Shorea macrophylla* is flood-tolerant, and according to Kenzo et al. (2007), *S. macrophylla* is a drought intolerant species and should be planted in high soil moisture or clay-rich conditions to avoid drought. According to USDA Soil Taxonomy, the soil type of *S. macrophylla* area consists of sand, silt, clay, and loam texture, is also known as the Grey-White Podzolic soil group (Adanan et al., 2020; Perumal et al., 2015). Although the Grey-White Podzolic soil is not suitable for most agricultural activities since it is developed through intense weathering and has a friable structure that could promote the occurrence of soil erosion, *Shorea* species are adaptable in extreme conditions, and they can grow well in this soil type as well (Leysia, 2012; Perumal et al., 2013). Grey-White Podzolic soils are pale in colour with weak to strong subangular blocky texture (Adanan et al., 2020). According to an analysis conducted by Adanan et al. (2020) at Sabal Forest Reserve (Sarawak), they found out the Grey-White Podzolic soils are strongly acidic with high contents of exchangeable aluminium and low contents of exchangeable bases, with a clay content of 9.3% to 27.9% in different plots. Clay deposition adds to limited water retention, which may be linked to the occurrence of large floods in the habitat of *S. macrophylla* (Perumal et al., 2015).

MORPHOLOGICAL CHARACTERISTICS OF *SHOREA MACROPHYLLA*

Shorea is the largest member of the Dipterocarpaceae family, and it is the emergent or main canopy of the forest. The genus *Shorea* is classified into 11 sections,

each with its androecium characteristics (Ashton, 1982). The species within sections are defined principally by their leaf morphology and tomentum (Ashton et al., 1984). However, identifying species morphologically is challenging for *Shorea* species as the appearances of the species grouped under this genus are relatively similar (Rosdayanti et al., 2019). Several studies on the morphology of *S. macrophylla* have been carried out. However, there are differences in the identification of morphological characteristics among researchers in Malaysia and Indonesia. According to Hotta (1997), who conducted his research in West Kalimantan, *S. macrophylla* is a small tree with sub-persistent stipules, outside tomentose, with leaf midribs that are usually pubescence and a mature tree height of 15 to 20 metres. However, the tree size of *S. macrophylla* in Sarawak, Malaysia, according to Lee et al. (1997), is medium or large, reaching up to 50 metres in height, four metres in stem girth at breast height, and two metres in buttress height. The description of Lee et al. (1997) is consistent with the *S. macrophylla* species observed in the planted plot at Semenggoh Wildlife Centre under Sarawak Forestry Corporation. It has large leaves, elliptic-oblong, base obtuse or subcordate, 13 to 18 pairs of prominent, well-spaced nerves and hairy midrib above, petiole stout, bark smooth or shallowly scaly, bole short (Ng et al., 2002). They are arranged in alternate forms, with secondary and tertiary veins visible or a three-centimetre long leaf stalk with leaves around 40 centimetres long (Riska & Manurung, 2018).

Shorea macrophylla had a smooth and brownish-green bark in its early stages of growth, which progressively evolved into an elderly tree with cracking and flaking (Chai, 1998; Riska & Manurung, 2018). Windyarini and Hasnah (2015) describe the stipule shape of *S. macrophylla* as spear-shaped, blunt, or elliptic, with a length of 5 cm and a width of 1.3 cm. They also discovered that a ten months-old *Shorea* plant might grow to a height range of 67.19 to 88.79 cm with a diameter of 9.65 to 10.33 mm and sturdiness of 7.0 to 9.21 (Windyarini & Hasnah, 2015). Figures 1(A) and 1(B) show the images of adult *S. macrophylla* tree and their canopy view. According to Sidi et al. (2021)'s research on insect pest studies on *S. macrophylla*, foliage damage was primarily caused by hairy caterpillars from the Lymantriidae in the early stages of tree development, where the most prevalent foliage was 'hole damage.'

Researchers met difficulties in classifying *Shorea* species due to their large sizes, unpredictable flowering season, and the absence of reproductive structures most of the time. Furthermore, several closely related and morphologically similar species may co-occur, making it confusing to identify their species without using any molecular approach (Moura et al., 2019).

FLOWERING AND FRUITING OF *SHOREA MACROPHYLLA*

The flowering and fruiting systems of most Dipterocarps, including *S. macrophylla*, are irregular. However, according to Chai (1998) and Mathiesen (1994), *S. macrophylla*

(A) (B)

Figure 1. Diagram of the canopy of *Shorea macrophylla* (A) and adult *S. macrophylla* tree reaches a height of about 50 meters (B) taken from Engkabang plot in one of the natural reserve centres named Semenggoh Wildlife Center under Sarawak Forestry Corporation

flowering occurs between September and October, with fruit ripening between January and March. Flowering may begin around 15 to 16 years; however, it may take several years before the next flowering occurs (Chai, 1998). The Dipterocarp trees flower yearly, although the flowering and seed production is limited. However, once every few years, a massive flowering event yields many seeds (Chai, 1998). The mass flowering phenomenon might be due to the combination of an aseasonality forest and the flowering system of dipterocarps itself (Appanah, 1993). An aseasonal forest is defined as a forest that does not have a consistent annual dry season and receives an average annual rainfall of around 2,900 mm (Appanah, 1993; Chandler et al., 2021). Plant phenological patterns, such as flowering, fruiting, and leaf change, are always more regular in a seasonal area, such as tropical America, than in aseasonal zones of Southeast Asia (Appanah, 1993). According to Ng et al. (2002), a significant level of inbreeding revealed that mating occurs primarily among relatives or closely related species, possibly due to the bigger fruit of *S. macrophylla*. As evidenced by their living environment, which is prone to continuous flooding, *S. macrophylla* is tolerant of soil acidity and well-adapted to harsh living conditions (Perumal et al., 2013). *Shorea macrophylla* grew well in canopy gaps, according to Grubb (1977).

Perumal et al. (2021) conducted a study on the growth performance of

different seedling ages of *S. macrophylla* and found that the survival rate decreased with increasing seedling age, with the three months old seedlings outperforming the six-, nine-, and twenty-four-month-old seedlings throughout the experiment. Therefore, they recommend keeping the three-, six-, and nine-month-old seedlings as planting material while keeping the twenty-four-month-old seedlings planted during the unpredictable fruiting interval (Perumal et al., 2021). Due to the inconsistent flowering of *S. macrophylla*, various studies have been conducted to determine the optimum way for its plantation. For example, Lo (1985) used a cutting technique in which single-node leafy cuttings from node two with 1,200 ppm and 3,600 ppm indole-3-butyric acid (IBA) were found to root better than the control plant samples.

In a fertilizer study conducted by Perumal et al. (2019), all types of fertilizers are advantageous in raising the seedlings in a nursery; however, jellyfish fertilizer with nitrogen: phosphorus: potassium ratio of 13.1: 1.7: 0.03 was the best for all morphological quality features compared to two others fertilizers tested, which are the chemical fertilizer (control) and controlled-instant release fertilizer, except for the root to shoot ratio which it still reached the average root to shoot ratio values (Perumal et al., 2019). Furthermore, the jellyfish fertilizer, which has a high percentage of organic matter (approximately 81%), is important for enhancing the physical structure of soil and, as a result, increasing soil moisture (Perumal et al., 2019).

GENETIC DIVERSITY AND DIFFERENTIATION OF *SHOREA MACROPHYLLA*

According to Kanzaki et al. (1996), *S. macrophylla*'s genetic structure is characterised by moderate genetic diversity within species and populations, as well as high genetic differentiation within local populations. Their moderate genetic diversity may be due to their limited dispersion in their environment and the immovability of their heavy fruit. These ecological features are most likely to contribute to the low gene flow among the local population (Kanzaki et al., 1996). The measured population genetics statistics of the plantation population were lower than those of the other populations, according to Utomo et al. (2018). Nurtjahjaningsih et al. (2012) developed microsatellite markers for four species of *Shorea* plants using the microsatellite primer of *Shorea curtisii*. Their findings revealed that the genetic diversity of all the plants, including *S. macrophylla*, had high genetic diversity and the inbreeding coefficient fit the Hardy-Weinberg equilibrium. Besides, among the *Shorea* species, *S. macrophylla* is closely related to *S. gysbertsiana* (Nurtjahjaningsih et al., 2012).

USES OF *SHOREA MACROPHYLLA*

Timber or Reforestation Sources

Shorea macrophylla, also known as the Light Red Meranti, is a suitable timber supply commonly utilised for light construction work (Ng et al., 2002; Yunanta et al., 2014). However, this timber is not resistant to

exposed conditions and cannot be treated with preservatives, so it is only suitable for interior wall boarding in construction (Chai, 1998; Wong, 2009). Furthermore, Ismaili et al. (2016) showed that Engkabang was classified under-strength group SG6, which is close to *Acacia mangium*, which was grouped in SG5, and concluded that Engkabang is suitable for furniture making and other non-structural applications.

Its fast-growing ability is due to its soft to medium texture timber structure, so this species is ideal for reforestation. The tree can reach a diameter of 50 to 60 cm at breast height (dbh) in about 20 to 30 years and continue regenerating after 15 to 16 years on the plantation (Ng et al., 2002). According to Lee et al. (1997), the average height of *S. macrophylla* was 19 metres after 21 years of planting, beginning in May 1973 with the first evaluation.

Besides being fast-growing, *S. macrophylla* has other beneficial characteristics for reforestation, such as its ability to adapt to harsh conditions (for example, flooding) (Indriani et al., 2019). Furthermore, fast-growing trees are important for changing the microclimate and soil's physical and chemical qualities, reducing understory temperature and humidity fluctuations, and providing better light for seedling development (Nawar, 2012). Therefore, *S. macrophylla* is an excellent candidate for reforestation purposes. The only problem with using *S. macrophylla* as a reforestation subject is the difficulty in obtaining a huge amount of readily available seeds because their viability is limited and they are mainly found in forests, and sampling is more difficult than those available in nurseries (Chai, 1998). Based to Tsumura et al. (2011), the amount of tax paid for imported wood is determined by their classified group, with *S. macrophylla* belonging to the Red Meranti group being among the highest tax timber. However, some *Shorea* species have been misclassified, resulting in a lower tax rate paid than it should be. Although identifying *Shorea* wood can be done by those well-trained and experienced in a few days, DNA analysis is recommended for a more accurate and convenient identification among species (Tsumura et al., 2011).

Illipe Nut

Shorea species such as *S. macrophylla* produce illipe nuts, known as 'butter' in the forest, and are widely used as cocoa butter replacer fat (Nesaretnam & Ali, 1992). The term 'illipe' came from South India to describe the nuts of *Bassia* species of the Sapotaceae family, as well as the Mowrah nuts of *B. latifolia* (Nesaretnam & Ali, 1992). Since then, the name 'illipe' has come to be used to refer to any oil-bearing nut with similar characteristics (Nesaretnam & Ali, 1992). The illipe nuts are large and have long calyx, which looks like a badminton shuttlecock, and they have a high oil content of 45% to 70% (Chai, 1998; Nesaretnam & Ali, 1992). Therefore, they are cultivated by the locals for their daily needs and as raw materials for other commercialised products (Nesaretnam & Ali, 1992). However, locals do not plant this plant in large quantities;

instead, they are mostly found in the forest, primarily for local use, and some are for exportation.

Their size and oil content governed the value of nuts; a bigger nut with more oil content is more valuable (Chai, 1998). The oil is yellow in colour and solid in nature (Roslan et al., 2019). Illipe nuts have a fat level of 52% to 53.9%, and they are high in C16, C18, and C18:1, making them a viable substitute for cocoa butter (Nesaretnam & Ali, 1992). Because the seed is recalcitrant and has a short viability period (Lo, 1985; Tompsett, 1998), it must be collected as soon as it falls and immediately placed in water (temporarily stored) to avoid direct germination, or else the fat will be utilised for seed germination and the oil content of nuts will be reduced (Chai, 1998). Submersion of the nuts in water can cause the seed coat to crack, yet it can also protect the seed against insect attack (Chai, 1998). Therefore, the seeds are removed from their coatings and dried under the sun to reduce moisture content (Chai, 1998; Nesaretnam & Ali, 1992). The dried seed can be stored longer than usual and is usually used for oil extraction (Lo, 1985).

Several studies have been conducted to determine the suitability of the oil derived from illipe nuts, also known as 'Engkabang fat', for use in the food industry. According to a study by Nesaretnam and Ali (1992), Engkabang fat has properties similar to cocoa butter fat. They also made chocolate using Engkabang fat and palm-mid fraction (PMF), which is a fraction from a palm that is rich in palm olein (POP), which is also known as desaturated triacylglyceride, proving that it is a suitable alternative to cocoa butter equivalent (CBE) fat, which is useful to substitute with cocoa butter that has a high demand in the market. The liquid fraction of Engkabang fat was more enriched with unsaturated triacylglyceride (TAG), suggesting that it might be used to make margarine or as a raw material for cosmetic products (Yanty et al., 2013). Canola-Engkabang fat blends have thermophysical qualities similar to lard, according to Illiyin et al. (2013). The proportion of palmitic and stearic acids increases as Engkabang fat (EF) is added to canola oil (CaO), whereas the amount of oleic and linoleic acids drop, and this makes their blending to be similar to lard (Illiyin et al., 2013). Roslan et al. (2019) conducted a continuing study to determine the composition. Physical features of Engkabang fat and canola oil blending before and after transesterification with *Mucor miehei* lipase*;* and they concluded that the slip melting point (SMP) value of the blended canola oil with Engkabang fat that has been interesterified for six hours is most closely related to lard (Roslan et al., 2019). Since the illipe nuts have high commercial value as they are suitable to be used in the food industry, the only problem is the limited seed production due to the unpredictable flowering and fruiting season of the Dipterocarpaceae family. More research can be done to understand the flowering and fruiting system of dipterocarp, as well as the long-term storage of the nuts for future analysis or use.

Natural Feed for Animals

Wild boar, woodpeckers, foxes, rodents, freshwater fish, squirrels, and local reindeer eat the illipe nuts, which contain a high oil content with mostly beneficial unsaturated triglycerides (Chai, 1998). The *Shorea* fruit is commonly consumed by riverine carp species, such as the *Tor tambroides*, also known as mahseer or empurau, as part of their natural diet. *Tor tambroides* are riverine cyprinids classified as endangered (Kamarudin et al., 2017). They are in high demand and command a high market price of up to RM800 per kilogram, depending on their size, grade, and origin (Entri, 2013). The cooked empurau reached up to RM1,300 per dish (Harith & Hassan, 2011). The special taste of the *T. tambroides* might have been adapted from the consumption of Engkabang fruit (Redhwan & Komilus, 2021). Kamarudin et al. (2017) researched the performance of crude illipe oil extracted from *S. macrophylla* as a dietary lipid source for riverine cyprinid *T. tambroides*. Their findings revealed that increasing dietary illipe oil levels had no significant impact on survival, growth performance, feed utilisation, body indices, lean percentage of *T. tambroides* juveniles, or whole-body proximate composition of the fish. However, generally, saturated fatty acids (SFA) and monosaturated fatty acids (MUFA) in the fish were found to be significantly higher after the feeding, but polyunsaturated fatty acids (PUFA) in the fish were lower after the feeding compared to control groups (Kamarudin et al., 2017).

Because of its growing region in the forest near riverbanks, Engkabang fruit is mostly used as a feed for several fish species; however, the data on Engkabang fruit as the natural feed of other animals is limited. Since the Engkabang fruit might give the empurau a distinct flavour, a study can be done into the factors that might cause a special aroma or taste. In addition, the Engkabang fruit might be tested as a food source for other animals to determine whether it can cause changes or specific tastes, similar to the *T. tambroides*.

Moisturising Agent for Lipstick

Norazlin et al. (2015) researched using Engkabang as the softening agent that could improve lipstick formulation by promoting moisturising protection. The lipstick containing the Engkabang fat worked better than the control lipstick (that did not contain the Engkabang fat) in their study. Engkabang fat, which has a lower melting point, offers the lipstick a suitable hardness and better spread-ability. Furthermore, Engkabang fat can also prevent moisture loss twice better as the control lipstick formulations, comparable to commercially available lipstick.

Another research was done by Rahman et al. (2011), who compared the suitability of Engkabang fat and Engkabang fat ester as an ingredient for cosmetic applications. Based on the properties and characteristics of the fat, their findings showed that Engkabang fat is suitable for cosmetic use, while Engkabang fat esters outperformed Engkabang fat in terms of moistening impact (Rahman et al.,

2011). Furthermore, the hydration values of the formulations, including Engkabang fat and esters, were higher than the control (Gani et al., 2010). All this could be due to a polar head group and the long hydrophobic tail of the Engkabang fat esters, which can act as a humectant and occlusive agent. The occlusive characteristics keep the skin moisture by reducing evaporation by forming a layer on the skin (Gani et al., 2010). Therefore, if the availability of Engkabang fruits can be increased, they might be proposed as the ingredient in cosmetic and healthcare products, as customers might prefer plant fat over animal fat.

FUTURE PROSPECT

Shorea macrophylla is a versatile plant used as timber and reforestation sources. Its fruit, the illipe nuts, are high in fat, which can be used as cocoa butter replacer fat and as animal feed. However, the studies conducted on Engkabang are still quite limited regarding their flowering and fruiting cycle or the Engkabang fruit itself. Since the Engkabang can be used for reforestation purposes, more research on how to optimise their growth can be done to learn more about the reforestation of this plant species and their supplementary uses. Furthermore, the illipe nuts are believed to provide nutritious food sources for industrial or agricultural applications. They are still underexplored due to the difficulty in obtaining these nuts, as the bearing of the nuts occurs only once every few years. However, their uniqueness and potential commercial value are intriguing and worth exploring. The International Union for Conservation of Nature (IUCN) categorised *S. macrophylla* as vulnerable back in the year 1998; however, it is listed as the least concern after being revised in the year 2019, so more research and investigation such as composition and other potential uses of the illipe nuts should be done to understand *S. macrophylla* and the illipe nuts further.

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Potential of By-Product of *Kappaphycus alvarezii* Derived from Bioethanol Production as Biofertilizer in Growing of *Ocimum basilicum* in an Aquaponic System

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ABSTRACT

Nutrient recycling from biowaste is one of the sustainable approaches to managing waste. The aquaponic system is one of the nutrient recycling methods that can reduce water consumption and reuse the nutrient available in its ecosystem. The nutrient to fertilize the plant in aquaponic depends on the activities of microbes to convert the waste into the nutrient. To enhance the growth of the plants, some aquaponics systems still rely on chemical fertilizers. *Kappaphycus alvarezii* is one of the red seaweeds abundantly found in East Malaysia. After numerous processes such as carrageenan extraction, the biowaste derived from *K. alvarezii* still contains a nutrient that can be recycled. The present study explores the potential of *K. alvarezii* solid waste as fertilizer to grow *Ocimum basilicum* in an aquaponics system. In this study, the macro- and micronutrients in *K. alvarezii* solid waste were determined, and the prevalence of microbes in the aquaponics system was monitored using inductively coupled plasma-optical emission spectrometer (ICP-OES) and 16S metagenomic sequencing method, respectively. Based on the findings, the growth of *O. basilicum* supplemented with *K. alvarezii* biofertilizer was significantly higher than the negative control. For genetic expression study in *O. basilicum*, *cinnamyl alcohol dehydrogenase* (*CAD*), *phenylalanine ammonia-lyase* (*PAL*), and *cytochrome p450 reductase* (*CPR*) genes were upregulated. The *O. basilicum* is free from mycotoxin and heavy metals. Since *K. alvarezii* solid waste is rich with macro- and micronutrients, which are essential for plant growth and can enhance the growth of *O. basilicum*,

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K. alvarezii solid waste produced from bioethanol production could be a potential fertilizer.

Keywords: Aquaponic, basil, biofertilizer, seaweed solid waste, sustainable agriculture

INTRODUCTION

In June 2017, the world population was 7.60 billion, expected to hit 8.60 billion in 2030. According to a new United Nations report, around 83 million people are added to the world's population annually (United Nations Department of Economic and Social Affairs [UN DESA], 2017). As the population grows, world agriculture must raise the demand for crop and livestock products. Currently, most countries still rely on local agriculture for food. However, there is an issue in increasing crop production due to the limitation of local resources under existing technological conditions such as semi-arid areas and soils problem. As such, agricultural research, extension, and infrastructure must be developed to solve the existing problem (Hemathilake & Gunathilake, 2022).

Traditionally, soils are the base for crop production, and large farmable land is required to produce the crop. However, long-term agriculture has resulted in soil damage; thus, the soil is not suitable for agriculture purposes. Besides, environmental pollution has polluted the soil and raised food safety issues. This hydroponic gardening was introduced to replace the conventional farming method. The hydroponic method is growing plants without soil, and the plants obtain the nutrients from the growing medium. The growing medium has become the heated argument about whether the hydroponic is certified as organic or not. The hydroponic system depends on applying nutrients from the concoction of chemicals, salt, and trace elements. Thus, the aquaponics system was introduced to replace hydroponics (Nawaz & Farroq, 2021; Van Gerrewey et al., 2022).

Aquaponics is a combination of recirculating aquaculture and hydroponics in one production system. The aquaponics system does not require soil and chemical pesticides to grow the plants. Therefore, it is a sustainable and intensive food production system and can produce a higher yield than conventional agriculture methods. Furthermore, the aquaponics system is not limited by non-arable lands such as deserts, degraded soil, and sandy islands (Nelson, 2017; Suhl et al., 2016). Therefore, the aquaponics system could be the best future agriculture method for food production. However, although the agriculture system would supply the essential nutrients to grow the plants, the available nutrient might not be sufficient to produce all kinds of plants. Thus, fertilizer is needed to provide a complete nutrient to develop a healthy plant.

Fertilizer is the major significant component in increasing the agricultural product. The public has noticed the disadvantages of chemical fertilizers; therefore, farmers turn towards organic fertilizers. One of the organic fertilizers which have been marketed is seaweed fertilizer. Seaweed extract fertilizer has

been claimed to be beneficial for seed germination, root development, increased frost resistance, increased nutrient uptake, increased resistance to fungal disease, and higher yields (Arioli et al., 2015; Gelli et al., 2020). Seaweed extract was rich in plant growth regulators such as auxins, cytokinins, ethylene, gibberellins, and abscisic acid (Arioli et al., 2015; Benítez García et al., 2020; Ghaderiardakani et al., 2019; Moncada et al., 2022). Besides, seaweed is also rich in minerals and trace elements which is essential for plant development (Petropoulos et al., 2020). However, seaweed extraction creates solid waste as a by-product, considered environmental waste accumulated in the landfill sites. A large amount of waste is generated during extraction as seaweed only accounts for 3–4% of yield, and the remaining are the waste fractions. To reduce the amount of solid waste in the environment, *K. alvarezii* solid waste was recycled and fully reused after bioethanol production as biofertilizers in aquaponics to replace the chemical fertilizers in the market.

Ocimum basilicum is one of the famous aromatic herbs widely used in traditional medicine and culinary herbs. The fresh and dried leaves are widely used as a spice, and the essential oil extracted from the fresh leaves is also used as food aroma additives and in pharmaceuticals and cosmetic products (Shahrajabian et al., 2020). Furthermore, *O. basilicum* is traditionally used as medicine for headaches, coughs, diarrhoea, constipation, warts, worms, and kidney malfunctions (Sonmezdag et al., 2018).

This study investigates the remaining *K. alvarezii* solid waste produced from bioethanol production as a biofertilizer in growing *O. basilicum* in an aquaponics system. Besides, the food safety of *O. basilicum* grown in the aquaponics system was also evaluated. The genetic expression of the genes *CAD*, *PAL*, and *CPR* in *O. basilicum* was also studied.

MATERIALS AND METHODS

Kappaphycus alvarezii Waste Preparation

Kappaphycus alvarezii solid waste from bioethanol production (acid hydrolysis) was collected and rinsed with distilled water. The *K. alvarezii* solid waste was dried until a constant weight was obtained. The dried *K. alvarezii* solid waste was ground into powder form and stored at -20 °C until further usage.

Macro- and Micronutrients in *K. alvarezii* Solid Waste

The macro- and micronutrients in the *K. alvarezii* solid waste were analysed using Association of Official Analytical Chemists (AOAC) Official Method 999.11. The tested macro and micronutrients are nitrogen, phosphorus, potassium, calcium, sulphur, magnesium, boron, chloride, manganese, iron, zinc, copper, molybdenum, and nickel. All the test parameters were expressed in parts per million (ppm).

Aquaponic Design

The aquaponic is consists of a recirculating aquaculture system (RAS) and a hydroponic

system (HS). Water from the fish tank was directed to a settling tank to remove all the solid waste produced from the fish and uneaten food. The settling tank consisted of a pump, biofilter, and filtered water compartment. For HS, the growing bedding consists of a leca medium to support the plants' growth. The filtered water from RAS was directed to HS to supply water and nutrients for plant growth. The used water from HS will be directed back to RAS and form a recycling system. In this study, *Barbonymus schwanenfeldii* fish and *O. basilicum* were grown in the aquaponics system.

Water Parameters in Aquaponic System

Aquaculture tanks' water parameters were determined using LAQUA Twin Compact Meters (Japan) (pH, temperature, ammonia, nitrite, and nitrate), dissolved oxygen (DO) meter, and carbonate hardness meter once a week. All the test parameters were expressed in ppm. In addition, the mean value for water parameters between negative control, positive control, and experimental groups were analysed. The data were statistically different when *P*-value was less than 0.05. This study's statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software (version 20).

Microbes Identification in Aquaponics System

DNA Extraction. The water sample from RAS and HS were collected, and the microbes in the water samples were cultured in nutrient broth overnight. The microbes were collected by centrifugation, and the obtained pellet was used for DNA extraction. The microbes' DNA was extracted with G-spin™ Total DNA Extraction Kit (iNtRON Biotechnology, Korea). The steps were conducted according to the manufacturer's protocol. The extracted DNA samples were stored at -80 °C. DNA quality and purity were assessed using fluorescence quantification (Qubit® dsDNA BR Assay Kit, USA) and 0.8% agarose gel electrophoresis (AGE).

Library Construction. The DNA sample was normalised to 30.00 ng per reaction. Next, polymerase chain reaction (PCR) was conducted to obtain the targeted DNA fragments. The PCR product was purified using Agencourt AMPure XP (Beckman Coulter™, USA). All the steps were done according to the manufacturer's protocol. The libraries were validated using the Agilent 2100 bioanalyzer instrument (Agilent DNA 10000 reagents, USA). The samples were then sequenced by using HiSeq 4000 sequencer (HiSeq® 4000 SBS kit, Illumina, USA).

Data Analysis Pipeline. The samples that Illumina MiSeq sequences in 10k tags were then accessed by FastQC v0.11.3 to ensure the quality of the raw sequencing reads. Low-quality reads and bases (lesser than Qv20), ambiguous (Ns), and artefacts were removed. Reads with a longer than 480 bp were also removed based on the estimated hypervariable region size. Finally,

a collective of the high-quality reads was merged as tags for downstream analysis with minimum base overlapping of 15 bp.

The sample was then aligned with tags against the SILVA rRNA reference database, followed by alignment refinement. All the chimeric reads will be identified and removed before performing operational taxonomic units (OUT) analysis. The sample was then assigned sequences to the taxonomy outline using the *k*-nearest neighbour (knn) consensus and the Wang's approach. It also includes reading assignments to OTU. Finally, the obtained information was used to perform diversity analyses using Quantitative Insights Into Microbial Ecology (QIIME).

***Kappaphycus alvarezii* Biofertilizer on Aquaponic System.** Forty-five *B. schwanenfeldii* fish (≈ 20.00 g) and *O. basilicum* (150 germinated seeds) were divided into three groups: Group I — do not supplement with any fertilizer (negative control); Group II — supplemented with 5.00 g/L commercial seaweed fertilizer (positive control); Group III — increased with 5.00 g/L *K. alvarezii* biofertilizer (experimental group). *Ocimum basilicum* was germinated before being transferred to the HS. Commercial seaweed extract fertilizer and *K. alvarezii* biofertilizer were dissolved in Milli-Q ultrapure water with a 5.00 g/L concentration. The fertilizers were applied to the O. *basilicum* leaves once per week. The *O. basilicum* was grown for 60 days. On the 60th day, the *O. basilicum* was harvested, the length and weight of the *O. basilicum* were measured, and the number of leaves and colour of the plant were also recorded. The leaves of *O. basilicum* were stored at -80 °C for molecular genetic analysis.

The mean value for height, dry weight, and the number of leaves between negative control, positive control, and experimental group were analysed. The data were statistically different when *P*-value was less than 0.05. This study's statistical analysis was performed using SPSS software (version 20).

Genetic Expression of *O. basilicum* in Aquaponic System.

***Total RNA Extraction*.** Total RNA was extracted from frozen tissue samples with an easy-Blue™ Total RNA Extraction Kit (iNtRON Biotechnology, Korea). Extracted RNA samples were stored at -80 °C. The RNA quality and purity were determined using NanoVue Plus™ spectrophotometer (United Kingdom).

***cDNA Synthesis for qPCR*.** According to the manufacturer's instructions, total RNA (1.00 µg) was used for cDNA synthesis with the AccuScript Hi-Fi cDNA synthesis kit (Agilent, USA). The primers sequence is provided in Table 1. The qPCR was carried out in triplicate using Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix (Agilent, USA). The reactions were performed using an ABI StepOne™ Real-Time PCR Systems (Applied Biosystems, United Kingdom), with universal cycling conditions. The *CAD*, *PAL*, and *CPR* genes were selected

in the study. In addition, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as a housekeeping gene to normalise gene expression between samples (Table 1).

Table 1
List of primers used in the qPCR

Gene	Forward (5' – 3')	Reverse (5' – 3')
GAPDH	CACCGAGGATGATGTGGTGT	GCAAGATCAGCCTTGGCATC
CAD	CGATGGCAAACCAACCCAAG	AGTAGTGGTGCTGCCTGTTC
PAL	TAAATGGGACAGCAGTGGGG	TCAAGTGGTCCGTGAACTCG
CPR	GCAGCACAAGATGGCACAAA	CCATGCCCTTAGCATCACCA

***Mycotoxin Detection in* O. basilicum.** The *O. basilicum* was dried and ground into powder. The mycotoxins in *O. basilicum* were extracted with 10.00 mL acetonitrile (MeCN) containing 2.00% formic acid. The mixture was then centrifuged, and the supernatant was obtained. The sample was then purified, and the solvent was exchanged with methanol: water [MeOH: H_2O (50:50, v/v)]. For mycotoxins quantification, 10.00 µL of the sample was injected into the High-performance liquid chromatography (HPLC) column, which was heated to 45 °C. A total of 10.00 mM ammonium formate and MeOH were used as mobile phases with a flow rate of 300.00 µL/min. The sample was run for 21 minutes (including 5 minutes of equilibration). The mycotoxin determination method was adapted from the AOAC Official Method 991.31. The tested parameters are aflatoxin (B_1, B_2, G_1, G_2), deoxynivalenol (DON), zearalenone, T-2 toxin, fumonisin (B_1, B_2), and ochratoxin A. All the test parameters were expressed in parts per billion (ppb).

***Heavy Metals Detection in* O. basilicum.** The heavy metals in *O. basilicum* were determined using AOAC Official Method 999.10. The test parameters were arsenic, cadmium, lead, and mercury. All the test parameters were expressed in ppm.

RESULTS AND DISCUSSION

The *K. alvarezii* biofertilizer was rich in phosphorus, potassium, calcium, sulphur, magnesium, and chloride. The macro- and micronutrients in *K. alvarezii* solid waste are shown in Table 2.

In general, green plants can produce their food by photosynthesis. For photosynthesis to take place, it needs nutrients to facilitate the photosynthesis process. Besides, the nutrients are also needed for plant growth and reproduction (Sharkey, 2020). In this study, the *O. basilicum* could obtain the nutrients from *K. alvarezii* biofertilizer and aquaculture system. The nutrients needed by the plants are divided into macro- and micronutrients. Macronutrients (nitrogen, phosphorus,

Table 2

The macro- and micronutrients in K. alvarezii *solid waste*

Test parameters	Reading
Total nitrogen	0.40 ± 0.03 %
Phosphorus	6581.60 ± 1.13 ppm
Potassium	15303.00 ± 0.92 ppm
Calcium	9603.80 ± 0.28 ppm
Sulphur	146064.70 ± 845.63 ppm
Magnesium	1740.70 ± 0.07 ppm
Boron	12.00 ± 0.14 ppm
Chloride	16120.40 ± 0.14 ppm
Manganese	177.10 ± 0.07 ppm
Iron	37.00 ± 0.21 ppm
Zinc	206.10 ± 0.21 ppm
Copper	170.10 ± 0.21 ppm
Molybdenum	ND (< 0.10)
Nickel	3.01 ± 0.06 ppm

Note. Values = Mean ± standard deviation; ND = Not detected

potassium, calcium, magnesium, and sulphur) are the nutrients that are needed by the plants in relatively large amounts, whereas micronutrients (boron, chloride, iron, manganese, copper, molybdenum, nickel, and zinc) are the nutrients needed in trace amount (De Bang et al., 2020). *Kappaphycus alvarezii* biofertilizer was rich in phosphorus, potassium, calcium, sulphur, and magnesium, which are the macronutrients needed for the *O. basilicum* to grow. Furthermore, a significant amount of boron, chloride, iron, manganese, copper, nickel, and zinc was also found in *K. alvarezii* biofertilizer.

In *K. alvarezii* biofertilizer, the nitrogen content was 0.40 ± 0.03%, and molybdenum was not found. Although the nitrogen content in the supplemented *K. alvarezii* biofertilizer is relatively low and molybdenum was not found, the *O. basilicum* in the negative control, positive control, and experimental group were still growing healthily. The *O. basilicum* do not have any nitrogen deficiencies symptoms such as yellowing of older leaves, thin stems, and poor vigour (McCauley et al., 2011). Nitrogen is needed for building structures, photosynthesis, cell growth, metabolic process, and chlorophyll production. Both nitrogen and molybdenum needed by the *O. basilicum* were obtained from the aquaculture system. The nitrogen in the aquaculture system exists in the nitrate form, and the *O. basilicum* can

utilise a moderate amount of ammonia and even the free amino acid in the aquaculture system. For molybdenum, it is correlated to nitrogen function, where the plants use molybdenum to catalyse redox reactions with different forms of nitrogen. The absence of molybdenum in the plants could lead to nitrogen deficiency symptoms even with nitrogen (De Bang et al., 2020; McCauley et al., 2011). Thus, the low content of nitrogen and molybdenum in the *K. alvarezii* biofertilizer could be supplemented from the aquaculture system and can still grow healthy plants.

Water Parameters in Aquaculture Tanks

The water parameters (pH temperature, ammonia, nitrite, nitrate, dissolved oxygen [DO], and carbonate hardness) in the negative control, positive control, and experimental aquaculture tanks showed no significant difference. The water quality for the aquaculture tanks is shown in Table 3.

The aquaculture system's water quality also plays an important role in growing *O. basilicum*. In general, the pH in the aquaculture system would interfere with the plant's access to nutrients. The pH beyond the tolerance range could cause the plant to be unable to take up the nutrients in the water even though the desirable nutrients exist. The desirable pH in an aquaculture system is between 5.50 to 7.50 (Oladimeji et al., 2018). The pH in the negative control (7.32 ± 0.30), positive control (7.55 ± 0.14), and experimental group (7.58 ± 0.21) were in the desirable range, which allows the *O. basilicum* to grow. Furthermore, a pH lower than 6.00 will reduce the nitrifying bacteria activities to convert the ammonia to nitrate. When the ammonia level increases, it will lead to an unbalanced system which is stressful and toxic to the fish (Sahrawat, 2008).

Ocimum basilicum is a warm climate plant which grows in temperatures of 17 °C to 30 °C. The temperature for the three

Table 3
Water quality of the aquaculture tanks

Water parameters	Negative control	Positive control	Experimental
pH	7.32 ± 0.30	7.55 ± 0.14	7.58 ± 0.21
Temperature (°C)	23.80 ± 1.24	23.90 ± 1.04	23.90 ± 1.08
DO (ppm)	6.59 ± 0.34	6.52 ± 0.18	6.73 ± 0.06
Ammonia (ppm)	0.06 ± 1.78	0.06 ± 0.18	0.06 ± 0.18
Nitrite (ppm)	0.06 ± 1.78	0.06 ± 0.18	0.06 ± 0.18
Nitrate (ppm)	143.13 ± 10.33	151.38 ± 3.96	148.50 ± 4.38
Carbonate hardness (ppm)	71.88 ± 5.94	75.63 ± 3.20	71.88 ± 2.59

Note. Values = Mean ± standard deviation

groups was ≈24 °C. For *O. basilicum*, the optimum temperature was suggested at 25 °C. The *O. basilicum* grows at 25 °C resulting in higher volatile oil content and taller plant (Chang et al., 2005; Saha et al., 2016). Furthermore, the DO content in the negative, positive, and experimental groups fell between 6.52 ± 0.18 ppm to 6.73 ± 0.06 ppm. Plants require high DO content in the water (> 3.00 ppm); Low DO content might result in rotting of the root, which will cause the growth of fungus.

Ammonia, nitrite, and nitrate are nitrogen sources for plants. Three nitrogen sources are available; however, nitrate is the most accessible nitrogen form (B. Z. Wang et al., 1989; Raven et al., 1992) because ammonia and nitrite are toxic to the fish, thus, it should always maintain below 1.00 ppm (McCauley et al., 2011). The ammonia and nitrite content in the three groups maintained an average of 0.60 ppm in the study. The nitrate content in the three groups was found in the range of 143.13 ± 10.33 ppm to 148.50 ± 4.38ppm. The nitrate in the water served as the nitrogen source for the *O. basilicum*; thus, even though the biofertilizer did not contain a high amount of nitrogen, the *O. basilicum* was still able to obtain the nitrogen from the water.

The last important water parameter in the aquaculture system is carbonate hardness. The optimal level for the aquaponic is 60.00 ppm to 140.00ppm, and the carbonate hardness in the three aquaculture systems was between 71.88 ± 5.94 ppm to 75.63 ± 3.20 ppm. Carbonate hardness is correlated with the pH in the aquaculture system, where it acts as a buffer (or resistance) to lowering pH. When nitrification occurs, nitric acid will be produced, dissociating in water into two components (hydrogen ion and nitrate). When the hydrogen ion increases in the water, it will reduce the pH of the water. The presence of carbonate and bicarbonate would stabilise against the acidification caused by the nitrification process (Russell, 2009; Wurts & Durborow, 1992). Therefore, the optimal conditions of the water parameters in the systems can create a healthy ecosystem for the fish, vegetables, and bacteria.

Metagenomics Analysis

The total throughput for the sample is 49,146 raw reads or ~14.50 Mbp of data, as depicted in Table 4. Over 86.71% of the sequencing data were retained after the quality filtering and merging process (Table 5), indicating that the sequencing reads are of moderate quality.

Table 4

Total throughput for the water samples collected from recirculating aquaculture system (RAS) and hydroponic system (HS)

Sample	Read size distribution	Total reads	Total bases	GC%
Recirculating Aquaculture system	291-297	24,340	7,180,300	54.00
Hydroponic system	294-297	24,806	7,330,017	53.50
Total	-	49,146	14,510,317	-

Note. GC = Guanine-cytosine content

Table 5

The sequencing statistics for the samples from recirculating aquaculture system (RAS) and hydroponic system (HS)

Sample		Before pre-processing		After pre-processing	
		Number of reads	%	Number of reads	%
Recirculating aquaculture system (RAS)	Forward	12,170			
	Reverse	12,170			
	Total	24,340	100.00	10,533	86.71
		Read size (bp)	%	Read size (bp)	%
	Forward	3,565,810			
	Reverse	3,614,490			
	Total	7,180,300	100.00	4,464,931	
		Number of reads	%	Number of reads	%
Hydroponic system (HS)	Forward	12,403			
	Reverse	12,403			
	Total	24,806	100.00	11,901	89.42
		Read size (bp)	%	Read size (bp)	%
	Forward	3,646,483			
	Reverse	3,683,691			
	Total	7,330,173	100.00	4,562,491	

A total of 241 OTUs were identified and assigned with taxonomy information up to the genus level. The top 4 OTU in RAS and HS and their respective assigned taxonomy and abundance are shown in Table 6.

Table 6

Top 4 abundant microbe identified in recirculating aquaculture system (RAS) and hydroponic system (HS)

Sample	Taxonomy	Relative abundance (%)
Recirculating aquaculture system (RAS)	Aeromonas	39.47
	Clostridium_sensu_stricto_3	24.85
	Unclassified Pseudomonadaceae	13.63
	Unclassified Enterobacteriaceae	9.92
Hydroponic system (HS)	Bacillus	34.74
	Unclassified Pseudomonadaceae	29.03
	Unclassified Enterobacteriaceae	12.16
	Aeromonas	6.76

A comparison of the OTU richness at the genus level between the samples was plotted. Between the RAS and HS samples, it was observed that 34 OTUs were shared between them, while 83 and 90 OTUs were specific to the former and latter, respectively (Figure 1). The number of species in the group RAS was 117, and HS was 124. The number of species shared between groups RAS and HS were 34 (16.43%).

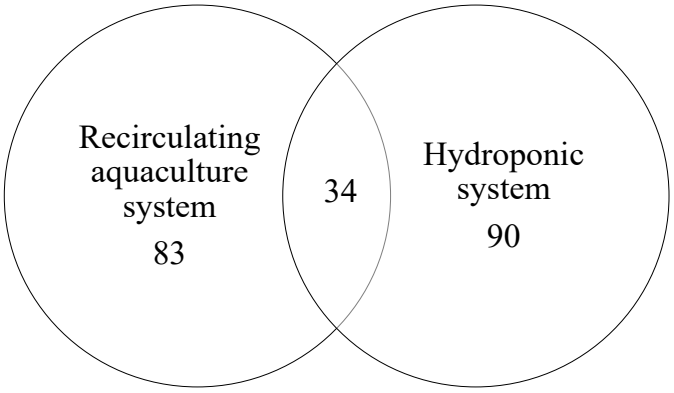

Figure 1. Venn diagram of OTUs at genus level between recirculating aquaculture system (RAS) and hydroponic system (HS)

A total of 241 OTUs bacteria were identified in the aquaponics system. The most abundant bacteria in the aquaponics system are *Aeromonas*, *Clostridium sensu stricto*, Pseudomonadaceae, Enterobacteriaceae, and *Bacillus*. These bacteria are heterotrophic and nitrifying bacteria that assist in converting fish waste into nitrate, an important nutrient for the plants (Chen et al., 2015; D. Xu et al., 2017a; He et al., 2016; Kathiravan & Krishnani, 2014; Y. Xu et al., 2017b) A total of 60.00–70.00% of the waste produced from the fish and released into the water is ammonia, the organic mix containing proteins, carbohydrates, fats, vitamins, and minerals. Nitrifying and heterotrophic bacteria metabolise the waste and convert it into macro- and micronutrients for the plants.

In the RAS and HS, the different prevalence of the bacteria observed is due to the changes in the environmental conditions which affect the attachment processes of the bacteria. The abundance of the bacteria depends on environmental conditions, such as changes in nutrient concentrations. Besides, the presence of the bacteria also depends on the characteristics and the species of the bacteria. Since the bacterial samples were collected from two environments, thus, the environmental conditions and nutrient contents were also different. Therefore, the prevalence of bacteria in these two systems was also different.

Ocimum basilicum Growth in Aquaponics System

The *O. basilicum* was grown in the aquaponics system and designated into negative control, positive control, and experimental group. After 60 days of growth in the aquaponics system, the height, dry weight, and the number of leaves in the positive control and experimental group were significantly higher than in the negative control group, with a *p*-value less than 0.05 ($P < 0.05$). The mean height, weight, and the number of leaves for *O. basilicum* are shown in Table 7. Furthermore, the *O. basilicum* appeared in healthy, bright green colour in all the groups.

Table 7

Mean height, dry weight, and number of leaves of O. basilicum *in the negative control, positive control, and experimental group*

Group	Height (cm)	Dry weight (g)	Number of leaves
Negative control	10.50 ± 1.15^{a}	0.71 ± 0.07^{a}	12.00 ± 2.11^{a}
Positive control	18.90 ± 0.88^{b}	3.59 ± 0.17^{b}	30.00 ± 1.69^{b}
Experimental	20.00 ± 1.41^{b}	3.73 ± 0.21^{b}	31.00 ± 1.69^{b}

Note. Values = mean ± standard deviation of n = 40 plants in each group. Different superscript letters in each row indicate a statistical significantly different at $P < 0.05$

The *O. basilicum* was grown in a stable aquaponics system. The height, dry weight, and the number of leaves of *O. basilicum* in positive and experimental groups were significantly higher than the *O. basilicum* grown in the negative group. Although the fish waste would be converted to nutrients by the bacteria, the nutrients produced in the water system might not be sufficient for the plants to grow. Besides, the conversion rate from waste to nutrients is slower than the uptake rate by the plants; thus, supplemented fertilizer would assist in plant growth (Bindraban et al., 2015; Han et al., 2016; Hussain & Abbasi, 2018; Liu et al., 2014). Therefore, the positive control and experimental groups were supplemented with a fertilizer, where the fertilizers are rich in macro- and micronutrients. Thus, *O. basilicum* grown in the positive control and experimental group was bigger than in the negative control group.

mRNA Expression in *O. basilicum* by qPCR

Three genes (*CAD*, *PAL*, and *CPR*) were selected to study the growth of *O. basilicum*. Compared to the negative control, *CAD*, *PAL*, and *CPR* genes were upregulated in the positive and experimental groups. *PAL* and *CPR* were not significantly different between the positive and experimental groups. However, *CAD* gene expression was significantly higher in the positive control group than in the experimental group. The genetic expression of *O. basilicum* is shown in Figure 2.

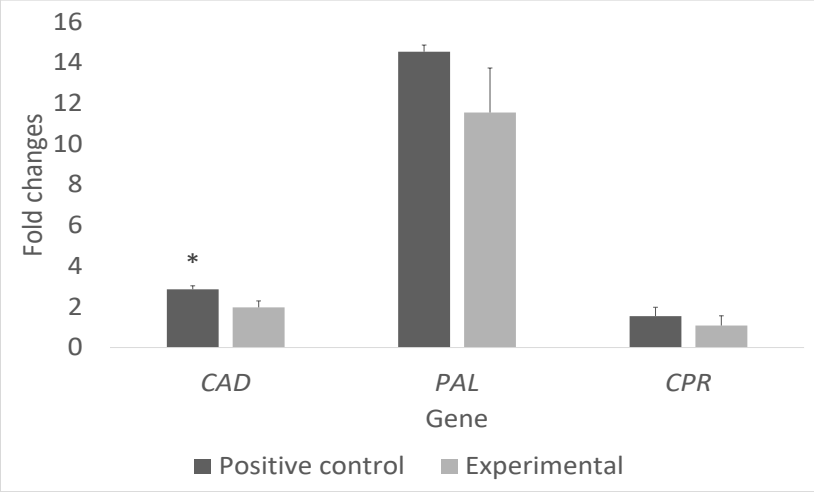

Figure 2. Fold changes in mRNA expression for the positive and experimental group on the 60th day. * in the bar chart indicate a statistical significantly different between positive control and experimental groups at $P < 0.05$. The x-axis indicates the genes of the *cinnamyl alcohol dehydrogenase* (*CAD*), *phenylalanine ammonia-lyase* (*PAL*), and *NADPH-cytochrome p450 reductase* (*CPR*)

In this study, the *O. basilicum* has been supplemented with commercial seaweed fertilizer and *K. alvarezii* biofertilizer, respectively. Thus, the expression of secondary metabolites in the *CAD*, *PAL*, and *CPR* will interfere. Both *CAD* and *PAL* genes are responsible for essential oil and phenylpropanoid production. The essential oil gives the plants good aroma and flavour (G. J. Wang et al., 2001; Iijima et al., 2006; Mandoulakani et al., 2017) and is an important source of aromatic and flavouring in food, industrial, and pharmaceutical product (Charles & Simon, 1990). The essential oil content in the plant depends on the development state of synthesising tissue and the metabolic process. The seaweed fertilizers applied to the *O. basilicum* are rich in polysaccharides, proteins, polyunsaturated fatty acids, pigments, polyphenols, minerals, and plant growth hormones. Thus, it might be able to increase cellular metabolism and positively affect the plant's conditions, such as root elongation and root formation.

Furthermore, the positive influence of cell metabolisms also improves bud and cell division to give better vegetative growth and increase the number of glands. On top of that, the hormones found in the fertilizers will also interfere with the growth stimulation, increasing photosynthesis's effectiveness. The increasing photosynthesis will protect the chlorophyll from degradation and enhance its content in the leaves. Thus, *CAD* and *PAL* genes were upregulated to produce secondary metabolites such as essential oil (Jamali et al., 2014; Tawfeeq et al., 2016). The expression of the *CAD* gene in the positive group is significantly higher than in the control group. The differences between the groups might be because of

the different seaweed fertilizers used. The genetic expression in the plants can be inferred by the surrounding conditions such as temperature, water conditions, light intensity, plant growth hormone, and the fertilizer applied (Lobo, 2008). The commercial seaweed extract fertilizer is rich in amino acids, cytokinins, mannitol, auxin, and vitamins. Thus, these factors might be why the expression level for the *CAD* gene in the positive group is higher than in the experimental group.

Expression of *CPR* correlates with the biosynthesis of aromatic and flavonoid metabolism in the *O. basilicum*. Therefore, upregulated expression of *CPR* might increase the production of the flavour of the *O. basilicum*, thereby increasing its value of it (Ayabe & Akashi, 2006). Besides, *CPR* is also proposed to be associated with ursolic and oleanolic acid production (Misra et al., 2017).

Besides, supplementation with the fertilizers leads to upregulation in the *CPR* gene. The effects of fertilizer on hydroxyl radical generated in the DNA resulted in strand breakage of DNA and caused a significant biological effect such as carcinogenesis, mutagenesis, and cytotoxicity. To counterpart the effect of hydroxyl radical thus, the flavonoid is generated. Flavonoids are well-known strong superoxide radical oxygen scavengers and singlet oxygen quenchers widely used as the therapeutic agent (Sorata et al., 1984). Besides, flavonoids also reacted with peroxy radicals, which take part in the termination of a chain reaction during the autooxidation of polyunsaturated fatty acids (Torel et al., 1986). Thus, the fertilizers do upregulate not only the flavonoids content but also phenolics content and vitamins in the plants (Osuagwu & Edeoga, 2013; Salama et al., 2015).

Mycotoxins and Heavy Metals in *O. basilicum*

Food safeness of *O. basilicum* was evaluated by determining mycotoxin and heavy metals contents. There was no mycotoxin aflatoxin (B_1, B_2, G_1, G_2), deoxynivalenol (DON), zearalenone, T-2 toxin, fumonisin (B_1, B_2), ochratoxin A, and heavy metals (arsenic, cadmium, lead, and mercury) detected in *O. basilicum* grown in the aquaponics system. The results for mycotoxins and heavy metals in *O. basilicum* are shown in Table 8.

Table 8
Detection of mycotoxins and heavy metals in O. basilicum

Test parameters	Unit	Reading
Aflatoxin (B_1, B_2, G_1, G_2)	ppb	ND (< 0.10)
Deoxynivalenol (DON)	ppb	ND (< 5.00)
Zearalenone	ppb	ND (< 10.00)

Table 8 *(Continue)*

Test parameters	Unit	Reading
T-2 toxin	ppb	ND (< 5.00)
Fumonisin (B_1, B_2)	ppb	ND (< 50.00)
Ochratoxin A	ppb	ND (< 0.50)
Arsenic	ppm	ND (< 0.01)
Cadmium	ppm	ND (< 0.10)
Lead	ppm	ND (< 0.10)
Mercury	ppm	ND (< 0.01)

Note. Values = Mean ± standard deviation; ND = Not detected

As the *O. basilicum* will be served as food thus, the safeness of *O. basilicum* to be consumed has become a concern. Therefore, this study investigated the common food-related mycotoxins and heavy metals in *O. basilicum*. In Table 8, all the mycotoxins and heavy metals parameters were not detected in the *O. basilicum*. The tested mycotoxins and heavy metals were aflatoxin (B_1, B_2, G_1, G_2), deoxynivalenol (DON), zearalenone, T-2 toxin, fumonisins (B_1, B_2), ochratoxin A, arsenic, cadmium, lead, and mercury. Mycotoxins are secondary metabolites produced by microfungi and bacteria, which are pathogenic to humans and plants (Gallo, 2001; Bennett & Klich, 2003). Exposure to mycotoxins can result in cancers, kidney toxicity, and immune suppression; thus, growing a mycotoxins-free plant is important.

The application of heavy metals contaminated fertilizer and water to the plants has raised food safety concerns in public. Food contaminated with arsenic, cadmium, lead, and mercury is dangerous and can harm human health even in low concentrations. Arsenic poisoning might bring complications in body organ systems such as renal, respiratory, and immune systems (Mohammed Abdul et al., 2015). Besides, the cadmium that humans consume will be retained and accumulated in the kidney (proximal tubular cells), which is toxic to humans (Bernard, 2008). Furthermore, mercury poisoning can lead to a nephritic syndrome in severe cases with hematuria and anuria (Bjørklund et al., 2017; Park & Zheng, 2012). Lastly, lead is the most hazardous and cumulative environmental pollutant through exposure to air, water, and food sources. Accumulating lead in the body could lead to human poisoning and death (Assi et al., 2016; Patra et al., 2011). Thus, it is important to ensure that the crops grown in agriculture are free from heavy metals. Aquaponics supplemented with biowaste from seaweed as biofertilizer could be a better design for agriculture because all the water parameters in the aquaponics system are under monitoring.

CONCLUSION

The *K. alvarezii* biofertilizer was rich in phosphorus, potassium, calcium, sulfur, magnesium, and chloride, which is suitable for growing *O. basilicum* in the aquaponics system. In the aquaponics system, nitrifying and heterotrophic bacteria are abundant, which are needed to convert the food waste into nutrients needed for the plant to grow. The *O. basilicum* supplemented with *K. alvarezii* biofertilizer has a significantly higher growth rate compared to the negative control. Furthermore, *CAD*, *PAL*, and *CPR* genes were found to upregulated express, which the genes were correlated to essential oil, and the development of *O. basilicum*. *Ocimum basilicum* grows in the aquaponics system and is supplemented with *K. alvarezii* biofertilizer is free from mycotoxin and heavy metals thus it is safe to be consumed as food. Therefore, *K. alvarezii* biofertilizer could be a potential biofertilizer that can assist in plant growth.

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Temporal Effects of the Combined Use of Cricket Frass and Eucalyptus Biochar on the Yield and Tissue Nitrate Content in Chinese Kale

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ABSTRACT

A greenhouse experiment was conducted to estimate the influence of various application rates of eucalyptus-derived biochar combined with cricket frass on the soil properties and soil N transformation, and, in turn, affecting both shoot biomass yield and nitrate (NO_3^-) contents of Chinese kale (*Brassica oleracea*). Two consecutive kale crops were grown to investigate the temporal effect of the combined amendments of cricket frass and biochar. Six rates of biochar, 0%, 0.125%, 0.25%, 0.5%, 1%, and 2% w/w in combination with 0.55% w/w of cricket frass, were applied only once at the start of the experiment in sandy loam soil. Shoot biomass significantly increased under treatments of 0.125% to 0.5% w/w in the first kale crop and 0.125% to 0.25% w/w in the second crop compared to the cricket frass alone. However, the higher rates of 0.25% and 0.5% w/w within the first and second crops decreased shoot biomass relative to their lower rates in each crop. Tissue NO_3^- concentrations of the first kale crop significantly decreased under all biochar rates, whereas the opposite effect was observed in the second crop. These contrasting effects of biochar on tissue NO_3^- concentrations were attributed to nitrification inhibition in the first crop and nitrification stimulation in the second crop. The 0.125% w/w rate of eucalyptus-derived biochar was, therefore, recommended to be combined with cricket frass to improve yield and reduce tissue NO_3^- content in the production of Chinese kale.

Keywords: Cricket faeces, *Eucalyptus* branch-derived charcoal, nitrification inhibition, nitrogen transformation, vegetable nitrate

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INTRODUCTION

Cricket keeping has risen in many countries due to a change in consumer habits. Their popularity has since moved into more mainstream urban communities where crickets were once consumed only in poor countryside locales (Hanboonsong et al., 2013). Cricket farms are distributed throughout Southeast Asia, including Thailand, Laos People's Democratic Republic, Cambodia, South Africa, the Democratic Republic of the Congo, and Kenya (Halloran et al., 2018). Cricket-keeping systems in Thailand have been reported to be the world's most advanced (Halloran et al., 2017), numbering over 20,000 farms (Hanboonsong et al., 2013). As a result, massive waste, particularly cricket excrement, termed cricket frass, is released from the farms at an average of 44 Mg/farm/year (Halloran et al., 2017). Mismanagement of these wastes creates the potential for both environmental pollution and human health risks.

Cricket frass is rich in plant nutrients, particularly nitrogen (N), reported at 2.3 - 2.6% (Darby et al., 2017; Halloran et al., 2017) significantly higher than that of poultry manure, reported at 1.7% N (Halloran et al., 2017). The cricket frass-derived organic N mineralizes quickly into NO_3^- due to its N-rich material content. Nitrate leaks into the ground and surface water resources via improper waste management practices. Consumption of water contaminated with NO_3^- may lead to severe human health risks, including cancer, methemoglobinemia, hyperthyroidism, diabetes, and blue baby syndrome (Santamaria, 2006). Recycling cricket frass to a soil amendment will mitigate environmental pollution and human health risks and save chemical fertilizer costs. While peasant farmers in Northeast Thailand have claimed that cricket frass improves rice yield (Halloran et al., 2017), experimental research into the agronomic benefits of cricket frass is required. Very few studies exist to date relating to the employment of cricket frass as a soil amendment. Treelokes (2013) determined that 15.6 Mg crick frass/ha adding into a Ultisol rendered increased yields of morning glory (*Ipomoea aquatica*), kale (*Brassica alboglabra*), and coriander (*Coriandrum sativum*); over organic fertilizers, like cattle manure and urban composts. Furthermore, Darby et al. (2017) demonstrated that 3.16 and 3.83 Mg cricket frass/ha increased sweet corn yields to 21.2 and 25.4 Mg/ha, respectively, while unamended soil produced only 16.3 Mg/ha.

These results indicate that the N-rich properties of cricket frass are suitable as soil amendments for the production of organic vegetables that are commonly subject to N deficiency (Hartz & Johnstone, 2006). However, the rapid nitrification rate of N-rich organic material, *viz* cricket frass, results in a high concentration of soil NO_3^-, producing high NO_3^- accumulation in vegetables (Umar & Iqbal, 2007). Therefore, lowering the nitrification rate is of critical interest in alleviating the resulting NO_3^- content in vegetables.

Biochar is a pyrolyzed organic material used to improve soil and plant

growth, particularly in organic vegetables (Suksawang, 2016). Most attractive to scientific study are the consequential biochar benefits in soil and plant improvement (Butnan et al., 2015; Deenik et al., 2011). Such biochar constituents include fixed carbon (C), ash, and volatile matter (Butnan et al., 2015; Deenik et al., 2011). Volatile matter-derived molecules, such as ethylene, acetylene (Spokas et al., 2010), polycyclic aromatic hydrocarbons (Borchard et al., 2014), and α-pinene (Clough et al., 2010), have been shown to play a critical role in the inhibitory effect on nitrifying microorganisms (Dempster et al., 2012; Spokas et al., 2010, 2011). Furthermore, the chemical characteristics of biochar-derived-organic molecules change chronologically once incorporated into the soil (Spokas, 2013). While these chronological changes of biochar promise to affect the NO_3^- accumulation in plants, the temporal effects of biochar as a nitrification inhibitor have not yet been reported.

It was hypothesized that (i) cricket frass would enhance plant growth and yield, (ii) increases in biochar rates would decrease nitrification rates and ameliorate plant tissue NO_3^- content, and (iii) biochar effects on nitrification inhibition and alleviation of plant NO_3^- accumulation would fluctuate with each application. Therefore, the objective of the current study was to evaluate the temporal effects of cricket frass with different biochar rates on vegetable yield and tissue NO_3^- content in two consecutive crops.

MATERIALS AND METHODS

Soil, Cricket Frass, and Biochar

The Roi-et soil series (isohyperthermic Aeric Kandiaquults) was collected at 0–15 cm depth from the Field Research Facilities of the Plant Science Section, Sakon Nakhon Rajabhat University, Sakon Nakhon, Thailand (17°11' 10.1" N, 104° 05' 17.3"E). The soil was air-dried and sieved through a 2 mm sieve for further use in the pot experiments. Soil properties before the start of the experiment are shown in Table 1.

Cricket frass was obtained from a cricket farm in Thailand's Sakon Nakhon province. Contaminant matters, such as dead crickets and body parts, were removed, as well as the remaining feeds. Biochar was produced from eucalyptus (*Eucalyptus camaldulensis*) because of its high content of volatile matter (Antal et al., 2000), a nitrification inhibitor source. The eucalyptus branches were pyrolyzed in a kiln modified from a 300-litre metal tank at approximately 450 °C for two hours and left to cool down for about six hours. The cricket frass and eucalyptus biochar were sieved through a 2 mm mesh. Characteristics of the cricket frass and eucalyptus biochar are presented in Table 1.

Greenhouse Experiments

The pot bioassays of two consecutive crops were conducted in greenhouse conditions from January to April 2021. The average air temperature throughout the experiment was 33.9 °C. The pot experiments were arranged in a randomized complete block design and

Table 1
Properties of soil, cricket manure, and biochar

Material	Proximate analysis			Soil particle distribution			Soil texture	BD	pH	EC	TC	TN	NH_4^+-N	NO_3^--N
	VM †	Ash	fC	Sand	Silt	Clay								
	------- (%) ------			--------- (%) ---------				(g/cm^3)	(1:10)	(mS/cm)	----- (g/kg) -----		----- (mg/kg) -----	
Soil	–	–	–	71.91	6.29	21.8	Sandy Loam	1.51	5.79	0.070	0.41	0.047	11.6	9.22
Cricket frass	–	–	–	–	–	–	–	0.32	7.92	15.85	376.0	39.1	1,099	147.0
Eucalyptus biochar	19.9	9.5	70.6	–	–	–	–	0.24	9.42	1.36	651.4	5.84	5.55	4.23

Note. †VM = Volatile matter; fC = Fixed C; BD = Bulk density; EC = Electrical conductivity; TC = Total C; TN = Total N

replicated three times. Seven treatments were evaluated herein: the unamended; and cricket frass in combination with biochar at 0%, 0.125%, 0.25%, 0.5%, 1%, and 2% w/w. Chinese kale (*Brassica oleracea*) was used as the test plant, as it is typically high in NO_3^-.

In the first crop, pots (d = 20.3 cm, h = 30.5 cm, v = 4,936 cm^3) were filled with 5 kg of dry soil. Afterward, 12.5 Mg/ha or 0.55% w/w (27.6 g/pot) of cricket frass, a recommended organic fertilizer for Chinese kale (Chakatrakarn & Jala, 2015), was applied to each amended pot. Biochar at the rates of 0% (0 g/pot), 0.125% (6.25 g/pot), 0.25% (12.5 g/pot), 0.5% (25 g/pot), 1% (50 g/pot), and 2% w/w (100 g/pot) was added accordingly. The cricket frass and biochar applied to each pot were computed using the soil weight basis with an initial soil bulk density of 1.51 g/cm^3. The cricket frass and biochar were applied only once at the beginning of the first kale crop. The mixtures were incubated at a 65% moisture level of the soil water holding capacity (WHC) or 19.04% w/w (0.93 L/pot) for 15 days before the Chinese kale was transplanted. A commercial variety of Chinese kale was seeded and nursed in a plug tray for 15 days. A single 15-day-old seedling, selected for its homogeneity and health, was transplanted to each pot. Each pot was weighed daily, and the soil moisture content was maintained at 65% WHC throughout the experiment.

The above-ground kale was cut at 50 days after planting and then oven-dried at 65 °C until the weights were constant and dry shoot

biomass was achieved. On the same day, the soil bulk density was measured. Fresh soil was immediately sampled for mineral N content. The soil in each pot was left to air dry. The air-dry soil was sampled and sieved through a 2 mm mesh for laboratory analyses. Subsequently, all pots were managed to achieve equivalent soil weight (4 kg/pot) to grow the second kale crop. The management of the second kale crop was consistent with the first crop.

Laboratory Analyses

Volatile matter, ash, and fixed C of biochar were analyzed following ASTM D7582-15 (American Standard of Testing Material [ASTM], 2012). In addition, biochar total C (TC) and N (TN) were determined on a TN analyzer (multi-N/C® 2100S, Analytik Jena, Germany).

Particle size distribution and soil texture were determined using the pipette method, and soil bulk density was assessed via the core method (Pansu & Gautheyrou, 2006). The pH and electrical conductivity of soil, cricket frass, and biochar were determined in the ratio (of these materials) to water at 1:10. Total C of both the soil and cricket frass was analyzed using the Walkley and Black method (Nelson & Sommers, 1983), and TN concentrations were measured using the micro-Kjeldahl method (Bremner & Mulvaney, 1983). Ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) extraction of soil and the frass was performed via the 2 M potassium chloride (KCl) solution and determined through the distillation method (Stevenson, 1983). Tissue NO_3^--N concentration of the Chinese kale was determined following the salicylic acid assay of Cataldo et al. (1975).

Data Calculations and Statistical Analyses

Calculations of nitrification rate and nitrification inhibition were modified from Sahrawat (1980) through the following equation: nitrification rate (%) = [NO_3^--N/(NH_4^+-N+NO_3^--N] × 100; and nitrification inhibition (%) = [(nitrification rate of cricket frass without biochar – nitrification rate of cricket frass with biochar)/nitrification rate of cricket frass without biochar] ×100.

A one-way analysis of variance (ANOVA) based on a randomized complete block design was used to evaluate the effects of each combination of cricket frass and the corresponding biochar rates on the selected soil properties, soil N transformation, and shoot biomass and tissue NO_3^--N concentrations of the Chinese kale. ANOVA was performed using PROC ANOVA with SAS software 9.1 (SAS Institute Inc., 2004). Multiple comparisons were performed employing Tukey's honest significant difference test. To identify the most important variables used to determine the tissue NO_3^--N concentrations of the kale in both crops, we employed principal component analysis (PCA) using the PROC PRINCOMP model. Significant differences in all statistical analyses were at $p \leq 0.05$.

RESULTS AND DISCUSSION

Soil Bulk Density, pH, Electrical Conductivity, and Total N

Lowered bulk density (BD) and higher porosity are distinguished properties of biochar in soil physical improvement, as evidenced in our current study, in which varied combinations of biochar with cricket frass significantly affected soil bulk density (Table 2). The 2% w/w inclusion of biochar in the first crop and 1% and 2% w/w inclusions in the second crop significantly decreased soil BD compared to cricket frass with 0% biochar ($CrF+BC_0$) (Table 3). Decreases in soil BD were a result of the dilution effect of biochar (Verheijen et al., 2010). Low BD of our biochar was shown at 0.24 g/cm^3 (Table 1), as similarly reported by Zhang et al. (2010); at 0.25 – 0.30 g/cm^3, while that of soil was higher (1.51 g/cm^3).

Biochar rates increased soil pH and EC in both crops compared to the cricket frass alone (Table 3). The alkalinity of ash components of biochar prompted a rise in soil pH (Yuan et al., 2011). Electrical conductivity is the measurement of the concentration of cations and anions in soil (Miller & Curtin, 2007). Therefore, increased soil EC with concomitant increases in the biochar rates was a consequence of the ash-derived ions.

Increases in pH and EC through cricket frass application, as seen in higher pH and EC in cricket frass alone than in unamended soil (Table 3), were accountable to the alkalinity and salinity properties of cricket frass (Table 1). Our results agreed with Azeez and van Averbeke's (2012) results,

Table 2

Analysis of variance of the effects of combined uses of cricket frass and eucalyptus biochar on soil properties and plant growth, yield, and tissue NO_3^- concentrations

Source of variance	df †	Soil								Plant		
		BD	pH	EC	TN	NH_4^+-N	NO_3-N	Total mineral N	NR	NI	Dry shoot biomass	Tissue NO_3-N
		(g/cm^3)	(soil: H_2O =1:10)	(mS/cm)	(g/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(%)	(%)	(g/plant)	(g/kg)
Amendment (A)	6	***	***	***	***	*	***	***	***	***	***	***
Time (T)	1	***	***	***	***	***	***	***	***	***	***	***
A × T	6	**	ns	*	ns	*	***	***	***	***	***	***

Note. * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$

+df = Degree of freedom; BD = Soil bulk density; EC = Soil electrical conductivity; TN = Soil total N; NR = Nitrification rate; NI = Nitrification inhibition; and Tissue NO_3-N = Tissue NO_3-N concentration in kale

which demonstrated that animal faeces, like poultry, cattle, and goat gained pH of 6.94, 9.11, and 9.75; and EC of 93.9, 18.6, and 65.0 mS/cm, respectively. The significantly higher pH in the first crop than in the second crop (Tables 2 and 3) may result from the effects of the solubility of ash constituents, particularly calcium compounds. Butnan et al. (2015) stated that the mechanism of soil pH increases through the solubility of ash-derived minerals. They further elucidated that dissolution of ash-derived quick lime (CaO) produced immediate increases in soil pH, as observed in the first crop of this study. Meanwhile, ash derived-calcium carbonate ($CaCO_3$) rendered the extent increases in pH in the second crop.

Both crops significantly increased TN under 2% w/w of biochar relative to cricket frass alone (Table 3). Furthermore, while N was volatilized via hydrogen cyanide (HCN), ammonia (NH_3), and dinitrogen (N_2) in the pyrolysis process (Wu et al., 2011); some N was deformed into a complex structure that could be resistant to loss (Usman et al., 2015), as can also be seen in the current study that TN of biochar was shown at 5.84 g/kg (Table 1).

Chinese Kale Biomass

There was a significant interaction of soil amendment × time on the shoot biomass of kale (Table 2). In the first and second crops, combined uses of cricket frass with biochar rates of 0.125% to 0.5% w/w and 0.125% to 0.25% w/w, respectively, resulted in significant increases in dry shoot biomass of kale over cricket frass alone (Figure 1). The respective increases in kale shoot biomass under these lower biochar rates may be attributed to increased soil pH (Table 3) and enhanced nutrient availability (Mengel & Kirkby, 2001). The proper soil pH for the growth of leafy vegetables is 6.0–6.8 (Ebesu, 2004). In the current study, soil pH amended with 0.125%–0.5% w/w in the first crop was 6.91–7.05, and that of 0.125% and 0.25% in the second crop was 6.96–7.01. Furthermore, the improved biomass achieved through the lower rates of biochar may be due to the contribution of essential plant elements in biochar ash (Butnan et al., 2015; Deenik et al., 2011). In the first crop, 0.25% w/w trended to decreased shoot biomass compared to 0.125% w/w. Meanwhile, in the second crop, 0.5% w/w significantly decreased shoot biomass relative to 0.25% w/w (Figure 1). This decline in shoot biomass might be due to excessive increases in soil pH (Table 3), leading to the diminution of micronutrient availability. Weil and Brady (2016) demonstrated that micronutrient cations, e.g., iron, manganese, zinc, and copper, are transformed into insoluble hydroxides and oxides under alkaline conditions, rendering plants' deficiency of these nutrients. In addition, the antagonistic effects of ash-derived cations resulting from excess biochar inputs could bring about the depression of kale shoot biomass (Butnan et al., 2015).

Table 3

Soil bulk density, pH, electrical conductivity, and total N in crops 1 and 2 as affected by the combined uses of cricket frass with different rates of biochar

Amendment †	BD (g/cm^3)				pH (soil: H_2O = 1:10)				EC (mS/cm)				TN (g/kg)			
	Crop 1		Crop 2		Crop 1		Crop 2		Crop 1		Crop 2		Crop 1		Crop 2	
Un	1.40	a ‡	1.66	a	6.57	f	6.74	e	0.138	d	0.143	d	0.45	c	0.46	c
CrF+BC_0	1.38	ab	1.55	b	6.82	e	6.89	d	0.364	c	0.296	c	0.59	b	0.51	bc
CrF+$BC_{0.125}$	1.42	a	1.54	b	6.91	d	6.96	d	0.379	c	0.280	c	0.64	ab	0.56	bc
CrF+$BC_{0.25}$	1.42	a	1.49	b	6.98	cd	7.01	cd	0.383	c	0.283	c	0.65	ab	0.56	bc
CrF+$BC_{0.5}$	1.34	ab	1.50	b	7.05	c	7.12	c	0.421	bc	0.332	bc	0.65	ab	0.57	bc
CrF+BC_1	1.27	b	1.43	c	7.20	b	7.31	b	0.476	b	0.385	b	0.69	ab	0.63	ab
CrF+BC_2	1.13	c	1.28	d	7.47	a	7.55	a	0.613	a	0.468	a	0.76	a	0.69	a
p-value	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
F test	***		***		***		***		***		***		***		***	
CV (%)	3.25		1.37		0.43		0.61		5.87		7.47		7.83		7.41	

Note. *** = $p \leq 0.001$

† Un = Unamended; CrF = Cricket frass; and BC_0, $BC_{0.125}$, $BC_{0.25}$, $BC_{0.5}$, BC_1, and BC_2 = Eucalyptus biochar rates of 0%, 0.125%, 0.25%, 0.5%, 1%, and 2% w/w, respectively

‡ Means within the same column followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey's honest significant difference test)

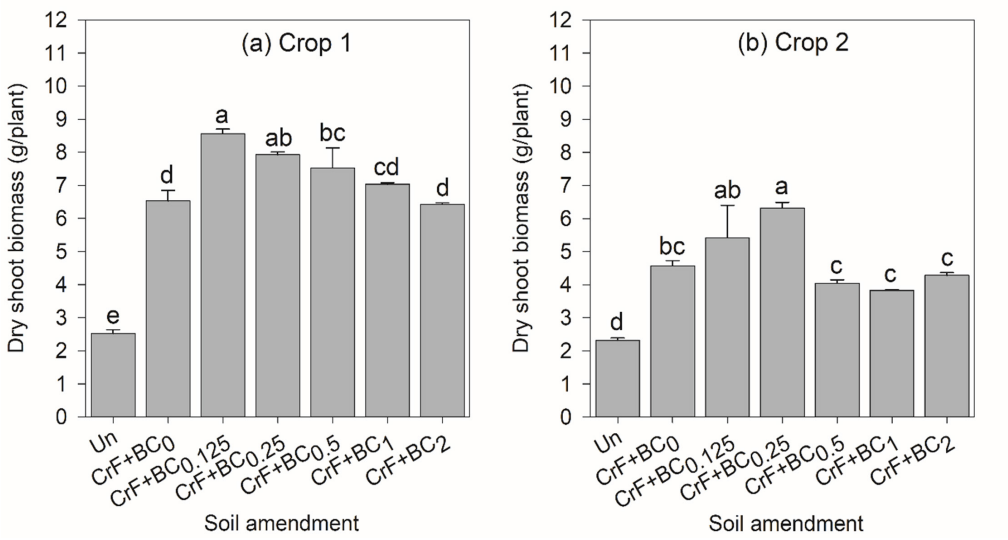

Figure 1. Dry shoot biomass of Chinese kale in the first crop (a) and second crop (b) as affected by the combination of cricket frass (CrF) with eucalyptus biochar at the application rates of 0% (BC_0), 0.125% ($BC_{0.125}$), 0.25% ($BC_{0.25}$), 0.5% ($BC_{0.5}$), 1% (BC_1), and 2% w/w (BC_2) in comparison with the unamended soil (Un); where bars with a similar letter indicate not statistically different ($p \leq 0.05$; Tukey's honest significant difference test), and error bars represent standard deviation (SD)

Soil N Transformation and Kale Tissue NO_3^- Contents

The biochar herein, derived from eucalyptus branches, acted as a nitrification inhibitor, alleviating tissue NO_3^--N concentrations of kale plants in the first crop and *vice versa* in the second crop. These contrasting effects are supported by the significant soil amendment × time interactions on soil NH_4^+-N and NO_3^--N concentrations, nitrification rates, nitrification inhibitions, and tissue NO_3^--N concentrations (Table 2).

All biochar treatments in the first kale crop significantly decreased soil NO_3^--N concentrations, bringing about significant decreases in tissue NO_3^--N concentrations (Table 4). Reductions in soil NO_3^--N concentrations in this cropping cycle resulted from the nitrification inhibitory property of biochar, as shown in the PCA (Figure 2). Soil NO_3^--N concentrations and nitrification rates were positively correlated with tissue NO_3^--N concentrations but negatively associated with nitrification inhibition (Figure 2a). An inhibitory effect of eucalyptus-derived biochar on nitrification in the first kale crop might be functioned by organic compounds constituted in the volatile matter of biochar (Dempster et al., 2012; Spokas et al., 2011). There were several volatile-derived compounds reported as nitrification inhibitors; for example, ethylene, acetylene (Spokas et al., 2010), polycyclic aromatic hydrocarbons (Borchard et al., 2014), and α-pinene (Clough et al., 2010).

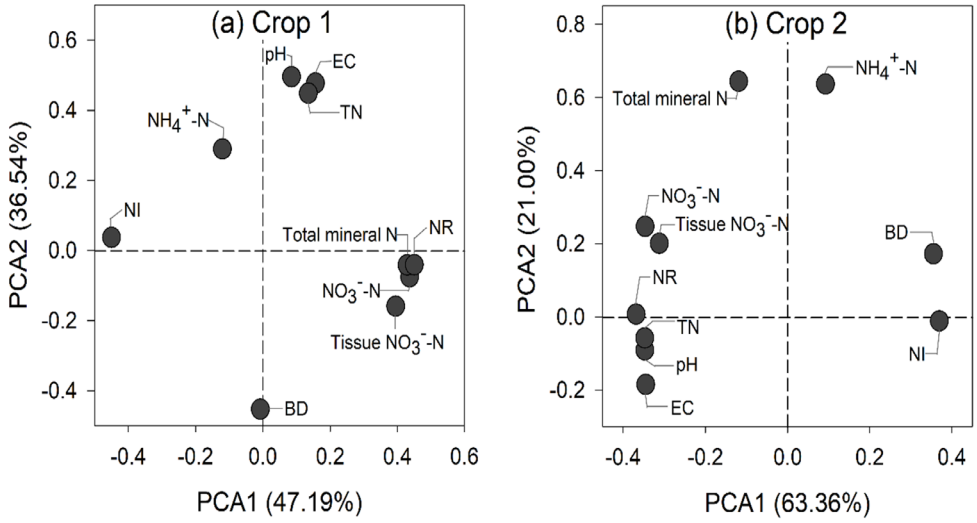

Figure 2. Eigenvectors of principal component analyses on relationships among selected soil properties, soil N transformation, and kale's tissue NO_3^--N concentration in crops 1 (a) and 2 (b)

Note. BD = Soil bulk density; EC = Soil electrical conductivity; TN = Soil total N; NR = Nitrification rate; NI = Nitrification inhibition; and Tissue NO_3^--N = Tissue NO_3^--N concentration in kale

In the second kale crop, biochar increased the nitrification rates (except for 0.5% w/w), resulting in higher NO_3^--N concentrations in the soil and plant tissues (Table 4). PCA results verified the biochar effects (Figure 2b), in which tissue NO_3^--N concentrations were positively correlated with NO_3^--N concentrations in the soil and kale tissues. In contrast, the results were negatively associated with nitrification inhibition. Therefore, biochar accelerated the nitrification rate instead of acting as an inhibitor (Table 4). Ethylene (C_2H_4), a volatile matter ingredient produced during pyrolysis and further formed after the biochar incorporation into the soil, might play a significant role in nitrification inhibition in this study. Spokas et al. (2010) determined that C_2H_4 in the volatile matter of biochar could inhibit soil nitrification. Fulton et al. (2013) further incorporated hazelnut shell-derived biochar containing 19.4% volatile matter into the soil, producing a volatile matter content similar to the levels observed herein. A large amount of volatile matter began to release from the soil on the first day of its incorporation and decreased abruptly. At 42 days after biochar incorporation, a minute amount of C_2H_4 was detected. Losses of C_2H_4 occurred within the first kale crop, bringing about no inhibitory effects of biochar on nitrification in the second crop. In addition to the loss of inhibitory effect of biochar on nitrification, increases in nitrification rates in the second kale crop might be due to the rise in nitrifier activity resulting from the elevated C and N contents in the soil mixture. These elements

Table 4

Soil mineral N concentrations and N transformation in crops 1 and 2 as affected by the combined uses of cricket frass with different rates of biochar

Amendment †	NH_4^+-N (mg/kg)		NO_3^--N (mg/kg)		Total mineral N (mg/kg)		Nitrification rate (%)		Nitrification inhibition (%)		Tissue NO_3^--N (g/kg dry shoot biomass)	
	Crop 1	Crop 2	Crop 1	Crop 2	Crop 1	Crop 2	Crop 1	Crop 2	Crop 1	Crop 2	Crop 1	Crop 2
Un	11.8 b-d ‡	4.81 ab	9.4 e	0.51 c	21.2 d	5.32 b	44.3 d	9.6 c	–	–	0.57 c ‡	0.52 c
CrF+BC_0	11.2 cd	3.65 c	44.4 a	0.68 c	55.7 a	4.33 c	79.2 a	16.0 bc	–	–	5.83 a	2.72 b
CrF+$BC_{0.125}$	13.7 a	4.89 ab	13.7 de	1.47 a	27.4 cd	6.36 a	49.9 cd	23.0 ab	+37.0 a	-43.4 ab	2.62 b	5.02 a
CrF+$BC_{0.25}$	10.8 d	4.78 ab	21.1 b-d	1.43 ab	32.0 bc	6.21 a	66.0 b	23.0 ab	+16.6 b	-43.6 ab	3.27 b	5.04 a
CrF+$BC_{0.5}$	12.9 a-c	5.28 a	27.3 b	1.14 b	40.2 b	6.42 a	68.1 b	17.7 bc	+14.0 b	-10.5 a	2.49 b	4.37 ab
CrF+BC_1	12.7 a-d	4.46 b	23.2 bc	1.64 a	35.9 bc	6.10 a	64.7 b	27.0 a	+18.3 b	-68.2 b	3.95 b	5.01 a
CrF+BC_2	13.4 ab	3.81 c	15.9 c-e	1.49 a	29.3 cd	5.30 b	54.3 c	28.1 a	+31.4 a	-75.6 b	0.50 c	4.72 a
p value	0.041	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.004	< 0.001	< 0.001
F test	*	***	***	***	***	***	***	**	***	**	***	***
CV (%)	8.44	7.67	23.27	15.21	16.83	7.07	4.50	14.64	13.05	-30.38	20.2	15.24

Note. *= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$

† Un = unamended; CrF = cricket frass; BC_0, $BC_{0.125}$, $BC_{0.25}$, $BC_{0.5}$, BC_1, and $BC_{2\,=}$ eucalyptus biochar rates of 0%, 0.125%, 0.25%, 0.5%, 1%, and 2% w/w, respectively

‡ Means within the same column followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey's honest significant difference test)

were not beneficial to the nitrifiers, possibly because the overriding effect of the C_2H_4 was abundant in the first kale crop.

Lower concentrations of soil NH_4^+-N, NO_3^--N, TN, and nitrification rate in the second crop compared to the first crop (Table 4) since the cricket frass and biochar were applied to the soil once only at the beginning of the first crop.

CONCLUSION

The results of this study showed that 0.125% to 0.5% w/w of eucalyptus branch-derived biochar in the first crop and 0.125% to 0.25% w/w in the second crop enhanced the shoot biomass of Chinese kale. Notably, increases in biochar amendments (0.25% and 0.5% w/w) in the first and second crops brought about decreases in shoot biomass.

All biochar rates reduced tissue NO_3^- concentrations of kale in the first crop but not in the second crop due to the inhibitory effects of biochar on nitrification in the first crop and, in contrast, the stimulation effect within the second crop. The most suitable rate of eucalyptus biochar in combination with 12.5 Mg/ha of cricket frass to enhance yield and alleviate tissue NO_3^- contents was 0.125% w/w or 2.831 Mg/ha. Additional applications of biochar in subsequent cropping cycles for eradicating nitrification stimulation and ameliorating tissue NO_3^- content are necessary for further investigation.

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Case Study

Radiographic, Computed Tomographic, and Cellular Phenotypic Features of Primary Nasal Transmissible Venereal Tumors in Four Dogs

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ABSTRACT

The primary nasal canine transmissible venereal tumor (CTVT) is a rare disease that develops by the allografted transmission of neoplastic cells in the nasal cavity. The disease is uncommonly reported in free-roaming dogs, with the social behavior of excessive licking and vigorously sniffing the affected parts of the other dogs in an endemic population. Post-chemotherapeutic computed tomography (CT) scan features and correlation of vincristine sulfate with cellular phenotypes have been scarcely reported in previously available primary nasal CTVT studies. This study describes the radiographic, computed tomographic, and cellular phenotypic features in four dogs affected with stage-4 intranasal CTVTs. The post-chemotherapeutic features of the nasal cavity in fully recovered cases are also highlighted. All data were analyzed retrospectively. All four dogs had stage 4 modified Adam's staging for nasal tumors due to the complete or partial lysis of the cribriform plate and lymphocytoid plasmacytoid (mixed) phenotype of the neoplastic cells based on the cellularity of cytological samples. All four dogs responded well to five cycles of vincristine sulfate and recovered completely. Two out of four dogs have follow-up scanning after chemotherapy. Based on the present study results, vincristine sulfate is still an effective monotherapy to achieve full recovery, although the number of cycles can vary, possibly depending on the expressed phenotype. Permanent loss of nasal turbinates is the sequelae of therapeutic chemotherapy. Prognosis is not correlated to the staging system but seems good with vincristine sulfate in mixed phenotype cases.

Keywords: Canine transmissible venereal tumor, cellular phenotype, computed tomography, modified Adam's staging, vincristine sulfate

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INTRODUCTION

The canine transmissible venereal tumor (CTVT) is also known as sticker's sarcoma, transvenereal sarcoma, infectious sarcoma, canine condyloma, transmissible lymphosarcoma, venereal granuloma, and contagious venereal tumor (Das & Das, 2000; Nemzek et al., 2015; Thamm, 2007). Despite immense mutation, CTVT adapted, survived, and spread across multiple continents, making it the oldest known continuously passaged somatic cell line (Nemzek et al., 2015). CTVTs are distributed globally, predominantly in tropical and subtropical regions. It is a naturally occurring, horizontally transmitted, parasitic-like, infectious neoplasia of dogs (Ganguly et al., 2013; Strakova & Murchison, 2014; Thamm, 2007; Ujvari et al., 2017). Due to the uncertainty of its origin, previous studies have described it as lymphatic, reticuloendothelial, macrophage or myeloid, and histiocytic origin tumors. Other than this ambiguity, it is considered one of the dogs' most common round cell tumors (Albanese et al., 2002; Gimeno et al., 1995; Mukaratirwa & Gruys, 2003). The most common transmission mode is coitus, although it can spread through biting, licking, and sniffing the neoplastic sites of the dog's body (Conte et al., 2022; Das & Das, 2000; Ganguly et al., 2013; Thamm, 2007).

Exfoliation of living neoplastic cells and allogenic transplantation across abraded mucosa are mandatory for the development of genital or extragenital TVT (Nemzek et al., 2015; Strakova & Murchison, 2014). Studies on experimental transplantation stated three distinct phases of CTVT growth: (1) progressive, (2) static, and (3) regressive phases (Mukaratirwa & Gruys, 2003). CTVT cells avoid detection by the immune system due to the downregulation of the major histocompatibility complex I (MHC-I) via secretion of inhibitory cytokines such as transforming growth factor-beta 1 (TGFβ-1) and interleukin 6 (IL-6) molecules, and no MHC-II activity (Foster, 2016). Little MHC class I or II expression, was detected in an experimental model during the initial proliferative phase of tumor growth. However, at 12 weeks, MHC expression increased remarkably, further stimulated by lymphocytes, resulting in tumor regression (Foster, 2016). In previous studies, spontaneous regression due to the immune response (immunoglobulin G formation, lymphocyte-mediated cytotoxicity) has been documented at the tumor age of 2 to 9 months (Das & Das, 2000; Foster, 2017a; Nemzek et al., 2015; Thamm, 2007; Ujvari et al., 2017).

In the past, some studies over-represented the male or female populations. Nonetheless, in recent case reports and a survey study conducted on a larger scale, gender bias was not observed (Kabuusu et al., 2010; Strakova & Murchison, 2014). In addition, adult and intact dogs of reproductive age (2 to 8 years) are more prone to this disease, although there are some reports on neutered and 11 years old dogs (Ganguly et al., 2013; Kabuusu et al., 2010; Rogers et al., 1998; Thamm, 2007).

Extragenital primary sites have been documented for CTVT in previous studies. The nasal cavity is the second most common site (5% to 13% of all CTVT cases), followed by the skin, oral cavity, eyes, and rectum. Additionally, it is over-represented in adult male dogs (Brandão et al., 2002; Ganguly et al., 2013; Ojeda et al., 2018; Rogers et al., 1998; Thamm, 2007). The most common historical findings associated with the nasal form of CTVT are sneezing, snoring, inspiratory dyspnea, bilateral epistaxis, sanguinopurulent nasal discharge, submandibular/mandibular lymphadenopathy, nasal deformation, and soft fleshy swelling at the level of the nasal bone area (Levy et al., 2006; Ojeda et al., 2018; Rezaei et al., 2016; Sukhbir & Sood, 2016; Veloso et al., 2018).

Grossly, the tumor can be soft to firm in consistency and single or multinodular, sessile, or pedunculated, with or without ulcerative surface in both genital and extragenital form. Tumor size can be up to 15 cm in diameter in the genital form of the disease (Foster, 2017a, 2017b). Microscopically, plasmacytoid, lymphocytoid, and mixed phenotypes have been described in detail in previous studies (do Amaral et al., 2007; Setthawongsin et al., 2017). Generally, it is a large but uniform and round to ovoid or polyhedral-shaped cell tumor resembling lymphocytes, and its cytoplasm is pale blue with distinct peripheral cytoplasmic vacuolization. Binucleation, mitotic figures, and single or multiple nucleoli are often observed in cytology samples (Foster, 2016, 2017a; Thamm, 2007). A definitive diagnosis can be made based on the cytological findings of sterile cotton swab samples, impression smear, fine-needle aspirated samples, and histopathology of the biopsy tissue. Polymerase chain reaction (PCR) of the rearranged long interspersed nuclear element (LINE)-1/c-myc gene can be a suitable option in cases where the definitive diagnosis is difficult based on cytology or histopathology results (Ojeda et al., 2018; Thamm, 2007).

Cytological features of the tumor are uniform discrete round to polyhedral-shaped cells with moderately abundant pale blue cytoplasm and an eccentrically located nucleus, with occasional binucleation and mitotic figures. The most characteristic feature is the presence of numerous discrete clear cytoplasmic vacuoles.

Histopathology of TVT reveals compact masses of round or polyhedral cells with slightly granular, vacuolated, and eosinophilic cytoplasm. The neoplastic cells are arranged in a diffused pattern and supported by a thin trabecula of fibrovascular tissue (Thamm, 2007).

Radiographs are helpful for the observation of nasal deformities, including soft tissue or fluid opacity (radiopaque) of the nasal cavity and frontal sinuses (Levy et al., 2006; Rezaei et al., 2016; Sukhbir & Sood, 2016). A computed tomography scan is currently the best available supportive modality to see the aggressiveness of the nasal cavity and frontal sinuses changes, including bone lysis and metastasis in neighboring areas, before

and after treatment (Patsikas et al., 2018). Additionally, it is quite useful to perform CTVT staging by a modified Adam's staging system (Ojeda et al., 2018). Chemotherapy with vincristine or doxorubicin is the most common treatment used and suggested in previous studies of dogs diagnosed with the nasal form of the disease (Levy et al., 2006; Ojeda et al., 2018; Rezaei et al., 2016; Sukhbir & Sood, 2016). A fatal outcome and high metastasis risk (up to 5% in routine cases of CTVT) are documented in puppies and immunosuppressed adult dogs (Foster, 2016; Mukaratirwa & Gruys, 2003).

The present report describes the radiological, particularly computed tomographic, features of the disease in the canine nasal cavity during pre-and post-treatment phases. These features are rarely described in detail with the staging system, and information started emerging in recent publications with a limited number of cases from other parts of the world.

MATERIAL AND METHODS

The retrospective data of four dogs definitively diagnosed with primary intranasal CTVT at the University Veterinary Hospital (UVH) of Universiti Putra Malaysia were analyzed for history, diagnostics, clinical findings, and treatments related to the outcome. In addition, telephonic interviews were conducted with the owners regarding the current status of the dogs and any further history of tumor recurrence. All dogs were tested serologically for *Ehrlichia canis* with an enzyme-linked immunosorbent assay (ImmunoComb® Canine Ehrlichia Antibody Test Kit, Israel) and microscopically for other common blood parasites, such as *Babesia canis vogeli* and *Babesia gibsoni*, from peripheral blood smears. In addition, complete blood count (CBC) and selected parameters from the serum biochemistry such as protein level, renal, and hepatic panels, including electrolytes, were tested in all four cases.

Radiographs of the skull, mainly the nasal cavity, with left and right lateral and ventrodorsal views were taken for all dogs. Contrast-enhanced computed tomography (CT) images were acquired for all four dogs using a cone beam CT scanner (Fidex, Animage, USA). Moreover, the results of the "modified Adam's staging" criteria were used to stage the nasal tumor. The criteria were adapted from Adam et al. (2009), where stage 1 is confined to the unilateral nasal passage, paranasal sinus, or frontal sinus without any bony involvement beyond nasal turbinates. Stage 2 is confined to any bony involvement beyond nasal turbinates without evidence of orbital, subcutaneous, or submucosal mass. Stage 3 is confined to the orbit or nasopharyngeal or submucosal mass involvement, and stage 4 is indicated when the tumor causes lysis of the cribriform plate. Rigid rhinoscopy was performed in Dog-2. Follow-up CT scans were performed in Dog-3 and Dog-4 for three months after completing five intravenous (IV) chemotherapy cycles.

Tissue biopsy samples were collected by punch biopsy from Dog-1 only at the left maxillary gum caudal to the canine teeth. Impression smears and sterile cotton swab

samples of nasal secretions were collected for cytology and culturing of pathogenic bacteria and fungi, respectively, in all four dogs. Blood agar and MacConkey agar (HiMedia Laboratories Private Limited, India) were used for primary bacterial culture. In contrast, Sabouraud dextrose agar (HiMedia Laboratories Private Limited, India) was used to detect fungi in the samples. In addition, fine needle aspiration (FNA) samples were collected from the left side of the hard palate in Dog-1, soft swelling at the nasal bridge in Dog-2, intranasal soft tissue mass in Dog-3, and soft swollen mass at the right side of the hard palate in Dog-4.

RESULTS

Signalment

Two of the dogs were mixed breeds (Dog-2 and Dog-3), one Spitz (Dog-1), and another Siberian Husky (Dog-4). All dogs were young adult males. One was neutered, and the remaining three were intact. The mean ± standard error (range) age and body weight of these dogs are 2.5 ± 0.46 (1.5 to 3.5 years) and 18.4 ± 2.14 (12.4 to 22.4 kg), respectively. All four dogs were raised in a free-roaming lifestyle (Table 1).

Table 1

Age, sex, breed, body weight, remarkable clinical findings, and duration of clinical signs before the first presentation to UVH in four dogs diagnosed with primary nasal CTVT

Case no.	Breed	Sex lifestyle	Age (Years)	B. Wt (kg)	Primary complaint/s and remarkable clinical findings on first and subsequent visits	Duration of clinical signs
Dog-1	Spitz	M FR	2Y 3Mo	12.4	Occasional profuse bleeding or blood clots from nares. Serosanguinous discharge from left nares. 2-cm mass present at left maxillary gum with an ulcerative mark on the surface. Slight bulging at the level of the left lateral nasal bone surface. Left-sided epiphora	5-months
Dog-2	Mixed	MN FR	3Y 5Mo	22.4	Eleven (11) months before the current presentation recovered from genital-organ TVT with three vincristine cycles. Presented with bilateral epistaxis. Asymmetrical face because of the distorted nasal bone with a small soft lump on the dorsal surface. Right-sided epiphora with conjunctivitis. Small growth at the level of the right upper premolar. Discoloration of nasal planum. Left-sided sub-mandibular lymphadenomegaly	4-months

Table 1 *(Continue)*

Case no.	Breed	Sex lifestyle	Age (Years)	B. Wt (kg)	Primary complaint/s and remarkable clinical findings on first and subsequent visits	Duration of clinical signs
Dog-3	Mixed	M FR	1Y 8Mo	20	It started as unilateral epistaxis and turned into bilateral just one week before the presentation. Continuous blood mixed mucoid nasal discharge from left nares and occasional bleeding from right nares when the patient is subjected to physical stress. Prolapsed third eyelid of the left eye	5.5-months
Dog-4	Siberian Husky	M FR	3Y 3Mo	18.8	Epistaxis from both nares. Serosanguinous discharge from medial canthus of right eye without any sign/history of injury started one day before the presentation. Slight bulging-out of the right eye globe. Another bulging was noticed at the level of the right lateral nasal bone surface. A 1.5 × 1.5 cm mass observed on the right side of the hard palate	2-months

Note. B. Wt = Body weight; TVT = Transmissible venereal tumor; M = Male; MN = Male neutered; FR = Free roamer

Clinical Findings

The summary and duration of the detailed clinical signs before the first visit to UVH for individual dogs are summarized in Table 1. The most remarkable presenting complaint was chronic bilateral epistaxis in all four dogs, which started unilaterally in Dog-1 and Dog-3 and was reported bilaterally at the time of presentation. In addition to epistaxis, the second most remarkable finding was concurrent ocular manifestations in all four cases. Dog-1 showed left-sided mild epiphora, while Dog-2 and Dog-3 manifested right-sided epiphora, conjunctivitis, and left-sided prolapsed third eyelid. Dog-4 manifested serosanguinous discharge from the medial canthus, as shown in Figures 1 (a and b), and a popped-out right eye. A soft swelling-type bulging surface was noticed at the level of nasal bone in Dog-1, Dog-2 (Figure 1 [d and e]), and Dog-4 (Figure 1 [b]). Facial asymmetry was reported in Dog-2 due to distorted nasal bone surface. The same dog also noticed discoloration of the nasal planum and left-sided submandibular lymph node enlargement. Oral lesions were observed in three dogs. A 2-cm mass

Figure 1. (a, b) Dog-4 presented with epistaxis and serosanguinous discharge from the medial canthus of the right eye and bulging-out of the right dorsolateral nasal bone surface, diagnosed with primary nasal CTVT; (c) The same dog on follow-up, after successful treatment; (d, e) Dog-2 presented with epistaxis and soft swelling at the level of the dorsal nasal surface, causing asymmetry of the face, diagnosed with primary nasal CTVT; (f) The same dog on follow-up, after successful treatment

on the left maxillary gum, a small growth at the level of the right upper premolar, and a 1.5 × 1.5 cm mass on the right side of the hard palate were documented in Dog-1, Dog-2, and Dog-4. There were no other abnormalities or evidence of genitalia growth in the dogs during the initial presentation.

Hematology and selected biochemistry parameters were tested in all four dogs (Table 2). Cross verification of all hematology reports and examination of routine blood parasites were performed through blood smears. The most remarkable findings in hematology mean ± SE [reference range]) leukocytosis 24.14 ± 2.89 (6–17), with band neutrophilia 0.53 ± 0.21 (<0.3), segmented neutrophilia 16.57 ± 1.90 (3–11.5), and monocytosis 2.46 ± 0.71 (0.2–1.4) in all four dogs. Major changes in the biochemistry parameters are seen in total proteins 78.08 ± 4.96 (55–75), with hypoalbuminemia 24.83 ± 0.59 (25–40) and hyperglobulinemia 56.6 ± 5.74 (25–45), consequently reducing the albumin to globulin ratio 0.47 ± 0.07 (0.5–1.2).

Table 2

Hematological and biochemistry parameters of four dogs diagnosed with primary nasal CTVT

Parameters (units)	Dog-1	Dog-2	Dog-3	Dog-4	Reference range
Erythrocytes $x10^{12}$/L	6.84	6.59	7.34	6.54	5.5 – 8.5
Hemoglobin g/L	156	144	164	138	120 – 180
PCV L/L	0.35	0.38	0.45	0.35	0.35 – 0.55
MCV fL	51	58	61.6	54	60 – 77
MCHC g/L	446	379	363	394	320 – 360
CWCC $x10^9$/L	26.8	29.3	24.48	15.99	6 – 17
Band-neutrophils $x10^9$/L	0.54	0.88	--	0.16	< 0.3
Segmented-neutrophils $x10^9$/L	16.62	21.68	15.36	12.63	3 – 11.5
Lymphocytes $x10^9$/L	3.75	2.34	5.13	1.60	1.5 – 4.8
Monocytes $x10^9$/L	2.68	4.10	2.40	0.64	0.2 – 1.4
Eosinophils $x10^9$/L	3.22	0.29	1.55	0.96	0.1 – 1.3
Basophils $x10^9$/L	0	0	0.04	0	Rare
Thrombocytes $x10^9$/L	316	243	485	318	200 – 500
Sodium mmol/L	--	141	152	143	140 – 155
Potassium mmol/L	--	6.7	5.5	5.4	3.7 – 5.5
Chloride mmol/L	--	108	117	107	96 – 122
Calcium mmol/L	2.75	2.5	2.6	--	2 – 2.8
Inorganic phosphate mmol/L	--	1.3	1.63	--	0.8 – 2.5
Urea mmol/L	3.7	7.3	2.9	8.6	3 – 7.5
Creatinine µmol/L	103	92	79	85	88 – 176
Total bilirubin µmol/L	--	3	3	--	1.7 – 17
ALT U/L	35.4	25	41	22	5 – 90
ALP U/L	--	70	72	--	40 – 100
GGT U/L	--	--	9	--	0 – 11
Total Protein g/L	68	82.5	72	89.8	55 – 75
Albumin g/L	--	24.4	26	24.1	25 – 40
Globulin g/L	--	58.1	46	65.7	25 – 45
A:G Unit	--	0.4	0.6	0.4	0.5 – 1.2

Note. PCV = Packed cell volume; MCV = Mean corpuscular volume; MCHC = Mean corpuscular haemoglobin concentration; CWCC = Complete white cell count; ALT = Alanine transaminase; ALP = Alkaline phosphatase; GGT = Gamma-glutamyl transferase; A:G = Albumin: globulin ratio

Serologically, all dogs were tested for *E. canis*. Dog-1 was treated with doxycycline before the presentation and reported negative upon testing. Dog-2 showed a serologically high antibody titer (scale 5/6) and was treated with doxycycline in the initial management of epistaxis with poor outcomes. Dog-3 and 4 tested negatives for *E. canis* but managed with doxycycline with poor outcome in the initial course of the disease diagnosis while dealing with epistaxis.

Radiology and Rhinoscopy

A detailed summary of radiographic and CT findings with the staging of nasal tumors has been outlined in Table 3. All four dogs fell into stage 4 of the modified Adam's staging for canine nasal tumors due to the involvement of the cribriform plate. The radiographic appearance of neoplasia in Dog-2 is shown in Figure 2. The right lateral and ventrodorsal (VD) radiographs of the skull, including the nasal cavity, revealed an abnormally radiopaque (especially on the right side compared to the left in VD view) nasal cavity with loss of the nasal conchae and turbinates detail. In addition, the bony lysis of the cribriform plate is noticed in both lateral and VD views. The pre-and post-treatment CT scan appearances of the nasal passages of Dog-3 and Dog-4 are presented in Figures 3 and 4. Meanwhile, rhinoscopy examination of the nasal cavity in Dog-2 did not reveal the involvement of any foreign body in the nasal disease.

Table 3

Summary of radiographic and CT scan findings in all four dogs and staging of primary nasal CTVT

Case	Radiographic findings	Computed tomography findings at different regions of the nasal cavity			Modified Adam's staging
		Rostral region	Maxillary 4th premolar region	Frontal sinuses region	
Dog-1	Abnormally radiopaque nasal cavity in both lateral and VD views, partial loss of nasal turbinates, and cribriform plate detail	Soft tissue density filled the cavity with nasal bone and septum lysis	Lysis of septum, turbinates, and palatine bone. Soft tissue density was invading from right to left nasal passage	Soft tissue density filled (partial) right rostral frontal sinus, partial cribriform lysis	Stage 4
Dog-2	Abnormally radiopaque nasal cavity in both lateral and VD views, loss of nasal turbinates, and cribriform plate detail	Soft tissue density filled the cavity with nasal bone and septum lysis	Bilateral space-occupying soft tissue density involving right orbit also. Lysis of septum and turbinates	Soft tissue density in both frontal sinuses, prominent cribriform lysis	Stage 4

Table 3 *(Continue)*

Case	Radiographic findings	Computed tomography findings at different regions of the nasal cavity			Modified Adam's staging
		Rostral region	Maxillary 4th premolar region	Frontal sinuses region	
Dog-3	Abnormally radiopaque nasal cavity in both lateral and VD views, loss of nasal turbinates, and cribriform plate detail	Soft tissue density filled the cavity with nasal bone and septum lysis	Right orbit involved lysis of septum, turbinates, and palatine bone (orbital process). Soft tissue density invading from right to left nasal passage	Soft tissue density filled right frontal sinus, prominent cribriform lysis	Stage 4
Dog-4	Abnormally radiopaque nasal cavity in both lateral and VD views, loss of nasal turbinates, and cribriform plate detail	Soft tissue density filled the cavity with nasal bone and septum lysis	Both orbits involved lysis of the septum, turbinates, perpendicular plates (palatine), and the palatine bone. Soft tissue density filled both nasal passages	Soft tissue density in both frontal sinuses. Massive lysis of the orbital process, perpendicular plate (palatine), and cribriform	Stage 4

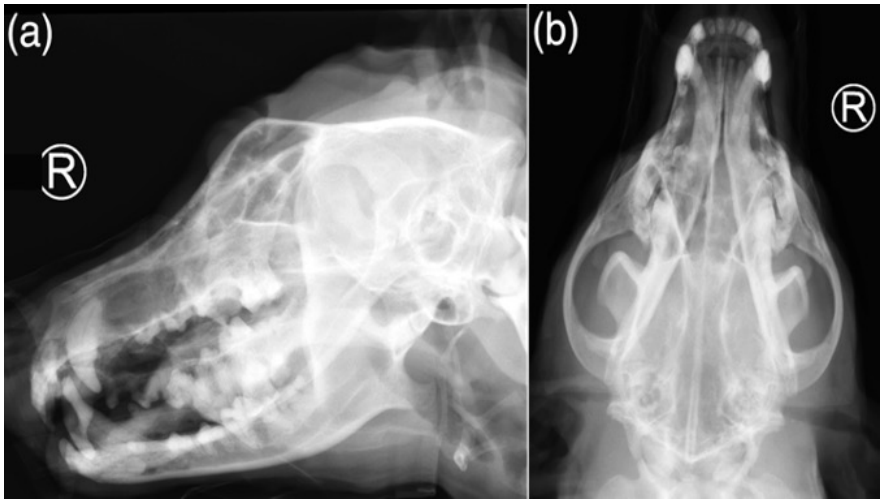

Figure 2. (a, b) Right lateral and ventrodorsal (VD) radiographs of the skull, including the nasal cavity, in Dog-2, diagnosed with primary nasal CTVT. An abnormally radiopaque (especially on the right side compared to left in VD view) nasal cavity with loss of the nasal conchae and turbinates detail and bony lysis of cribriform plate noticed in both lateral and VD views

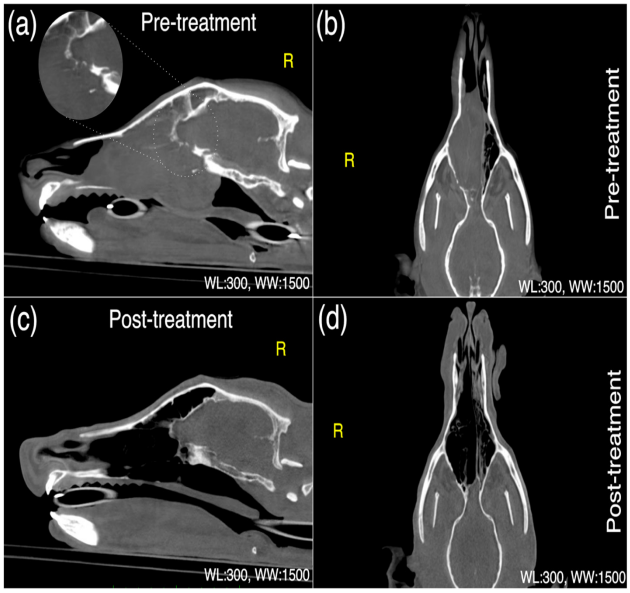

Figure 3. (a, b, c, d) Sagittal and dorsal pre-contrast bone reconstruction images of Dog-3 before and three months after chemotherapy. The right side is denoted by "R," and window level and width are labeled. (a, b) A large isoattenuating density occupies a major portion of the right nasal passage; (b) causing left-side deviation of the nasal septum and lysis of the cribriform plate [highlighted in (a)] including surrounding bony structures; (c, d) The nasal passage is clear post-chemotherapy. However, permanent loss of the nasal turbinates is observed, predominantly in the right nasal passage

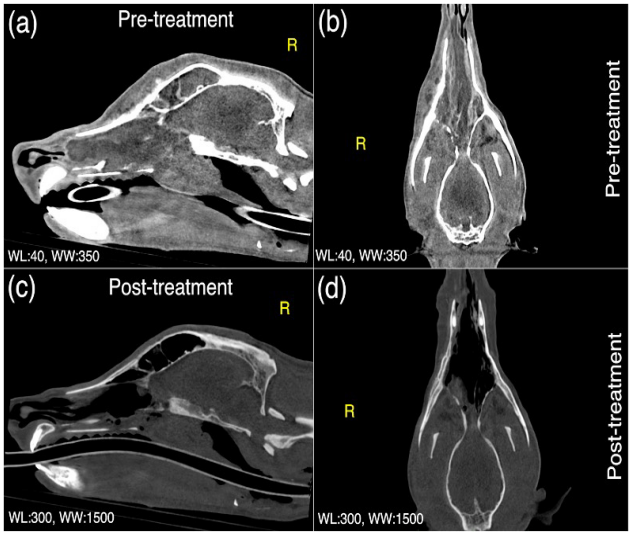

Figure 4. (a, b, c, d) Sagittal and dorsal pre-and post-contrast, soft tissue (a, b), and bone (c, d) reconstruction images of Dog-4 before and three months after chemotherapy. The right side is denoted by "R," and the window level and width are labeled. (a, b) A large mixed (iso, hyper, and hypo) attenuating density occupies the entirety of both nasal passages and (b) further infiltrates into right lateral nasal soft tissue; (b) causing significant left-side deviation of the nasal septum and lysis of the cribriform plate, including surrounding bony structures; (c, d) Nasal passages are clear post-chemotherapy. However, permanent loss of the nasal turbinates is noticed in both the left and right nasal passages

Cytology and Histopathology

Cytology was performed in all four dogs, and phenotypically, all samples exhibited identical findings. A mixed plasmacytoid and lymphocytoid cell population is noticed in impression smears and FNA samples of intranasal, paranasal, and invasive soft tissue swelling of the oral cavity. Characteristically, round to ovoid or polyhedral cells with distinct boundaries was noticed in all four dogs. Round nuclei containing prominent angular nucleoli and slight basophilic cytoplasm with distinct vacuolation were also observed. Mitotic figures were noticed in all four cytological samples, as shown in Figure 5.

Only Dog-1 had a histopathology report of a few pieces of whitish tissue measuring 1.8 × 1.5 × 0.2 cm in aggregate. Neoplastic cells identical to cytology samples were identified in the nasal mucosa arranged in sheets with fibrous septa, separating the tumor into vague nodules. Occasional mitotic figures and subepithelial lymphocytic infiltration were also observed in the histopathology samples.

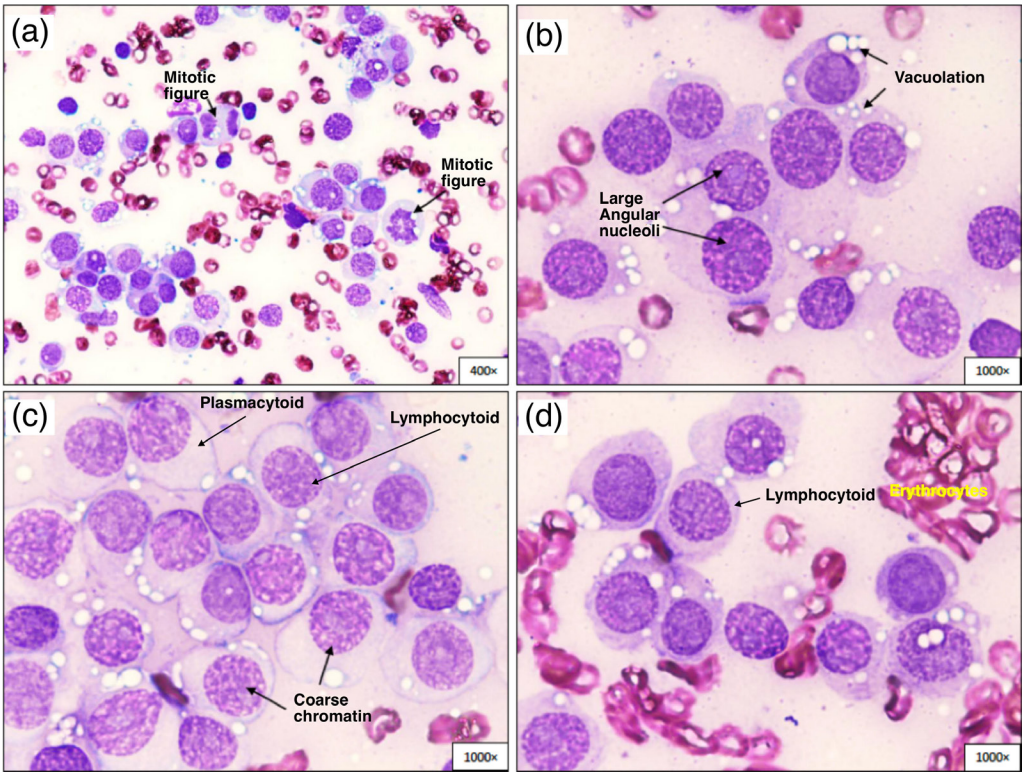

Figure 5. (a, b, c, d) FNA of the intranasal mass of Dog-3 was diagnosed with primary nasal CTVT. The phenotype is mixed type by classification characterized by plasmacytoid and lymphocytoid cell population (labeled). Several rounds to ovoid cells are observed with distinct cellular margins, cytoplasmic vacuolation, round nuclei with large angular nucleoli, and coarse chromatin. A mitotic figure is also noticed in this smear, as shown in section (a) 400 × (Modified Giemsa stain)

Treatment and Outcome

A detailed summary of treatment and outcome is presented in Table 4. All four dogs achieved complete resolution of clinical signs shown in Figures 1 (c and f) with vincristine sulfate (Vincristine Sulfate®, 1 mg/mL, Korea United Pharm. Inc., Korea) at a dose rate of 0.025 mg/kg q7days intravenously in five cycles without any obvious adverse reactions or side effects. Hematology was repeated at every follow-up for each case before administration of the chemotherapeutic agent. The most common finding was thrombocytosis in all cases. Furthermore, all dogs responded well to this therapy and are still alive without any recurrence of clinical signs, confirmed during subsequent follow-up post-treatment protocols, by detailed telephone interviews with the clients, and CT scans for Dog-3 and Dog-4. Follow-up scans were done after three months of the fifth cycle of vincristine. Though CT findings for Dog-4 at 3-month follow-up post fifth vincristine cycle are slightly questionable in remission scan (Figure 3), the density of the questionable tissue is homogenous, and it is quite caudal in the nasal cavity. On the other hand, owners were satisfied with the current remission state as the quality of life had improved significantly without any obvious clinical signs. Hence, cytology was not repeated on follow-up visits as constrained by clients. The last case in the present study was treated approximately seven months ago.

Table 4

Diagnostic confirmation, drug of the choice, outcome, and follow-up data of four dogs diagnosed with primary nasal CTVT

Case	Diagnostic confirmation	Treatment	Follow-up	Final outcome
Dog-1	Cytology (mixed) and histopathology	Vincristine chemotherapy, q7days for 5 weeks	Still alive after 37 months of last treatment without recurrence	Recovered
Dog-2	Cytology (mixed)	Vincristine chemotherapy, q7days for 5 weeks	Still alive after 19 months of last treatment without recurrence	Recovered
Dog-3	Cytology (mixed)	Vincristine chemotherapy, q7days for 5 weeks	Still alive after 10 months of last treatment without recurrence	Recovered
Dog-4	Cytology (mixed)	Vincristine chemotherapy, q7days for 5 weeks	Still alive after 08 months of last treatment without recurrence	Recovered

Note. Mixed = Phenotypically plasmacytoid and lymphocytoid cell population of cytological sample; q7 days = Quaque 7 days = Every 7 days

DISCUSSION

The most remarkable historical findings in the present study are the presentation of young adult male dogs with chronic (2–5.5 months duration) epistaxis and/or serosanguinous nasal discharge, which is quite consistent with previous case studies of primary nasal CTVT, where nasal discharge was reported from one month (Ojeda et al., 2018) to one year (Sukhbir & Sood, 2016). Although some concurrent oral and ocular manifestations were also noticed in all dogs during physical examination, these findings are also quite consistent with previously reported cases where allografted transmission causes an invasive nature of the disease spreading to surrounding areas of the nasal cavity (Komnenou et al., 2015; Veloso et al., 2018). In dogs, the top listed differential of epistaxis in tropical countries like Malaysia is canine ehrlichosis (Neer et al., 2002). In the present study, all dogs tested and went through standard therapy of doxycycline for at least four weeks, regardless of the serological results, and did not respond well.

Mild, microcytic, and non-regenerative anemia (packed cell volume [PCV] at low normal) was noticed in Dog-1, 2, and 4, likely due to prolonged bleeding resulting from chronic epistaxis, which agrees with the previous study of primary nasal CTVT (Papazoglou et al., 2001). Nevertheless, a similar finding was absent in Dog-3, where the PCV was at mid-range, possibly due to compensatory polycythemia induced by increased erythropoietin, endogenously produced by the tumor (Papazoglou et al., 2001; Rogers et al., 1998). All four dogs had mild-to-moderate leukocytosis, which could occasionally be present due to possible inflammation of the tumor surface (Ganguly et al., 2013).

Following the definitive diagnosis of these cases, CT findings (Table 3) revealed remarkable lysis of nasal turbinates, septum, and nasal bone in all four dogs. Palatine bone lysis is noticed in Dogs 1, 3, and 4, conclusive of oral cavity contamination with neoplastic tissue. In addition, the lysis of the orbital process of palatine bone is noticed in Dogs 2, 3, and 4, causing the manifestation of ocular signs. Since bony lysis is quite significant in all cases, the external and internal tables of the cribriform plate and frontal sinuses, separating sinuses from the nasal cavity, were also affected and allowed discharge and/or neoplastic tissue to spread into these areas. These findings indicated stage 4 of the modified Adam's staging used to stage the primary nasal CTVT in the present study. Staging with the same criteria has been done recently in a case study of four dogs diagnosed with primary nasal CTVT, where only one dog was categorized as stage 4 based on the CT scan findings. Interestingly, in the same case report, the aggressive nature of CT scan findings was noticed in the male population, whereas another two males were categorized as stage 3, and the only female was categorized as stage 1 (Ojeda et al., 2018).

The primary nature of the disease was considered based on the absence of genital form and/or metastasis of the primary tumor from another site, history, and clinical

findings related to nasal CTVT. Additionally, a free-roaming lifestyle and social behavior, such as excessive licking of genital organs of affected animals and vigorous sniffing habits, can be a possible mode of allografted transmission (Papazoglou et al., 2001). A definitive diagnosis can be cytologically made due to this study's typical characteristics of neoplastic cells. It can be further determined with PCR and histopathology analysis. In the present study, all four dogs were diagnosed mainly through cytology, while histopathology assessment was performed only for Dog-1. Contrary to a recent study on primary nasal CTVTs where the lymphocytoid phenotype was considered less aggressive than the plasmacytoid phenotype with aggressive lytic changes in the nasal cavity (do Amaral et al., 2007), the present study had a mixed phenotype (none of the samples demonstrated more than 60% single phenotype; Setthawongsin et al., 2017), with aggressive changes recorded from CT scans, as mentioned in the preceding paragraph (Table 3).

Although complete remission can be seen on follow-up CT images of Dog-3 (Figure 3 [c and d]), it is questionable in Dog-4. It is unknown whether the remaining soft tissue density lesion (Figure 4 [c and d]) comprises a residual biologically active tumor or the tumor is in a possible sterile or regressive state. It can be differentiated by performing positive emission tomography (PET) imaging (Lawrence et al., 2010); however, it is not regularly available in veterinary medicine. Alternatively, post-treatment sequential CT scans can be performed to evaluate disease progression, recurrence, and remission (Bowles et al., 2016). In addition, several studies have proven the evidence of nasal turbinate regrowth in cases of brachycephalic dogs undergoing laser-assisted turbinectomy (Schuenemann & Oechtering, 2014), pigs with atrophic rhinitis (Robertson et al., 1990), and cats and dogs following intranasal polyps and angioleiomyoma removal (Schuenemann & Oechtering, 2014). However, it remains uncertain, as a recent (Ojeda et al., 2018) and present study showed persistent lysis of turbinate and adjacent bones upon follow-up CT scans. Interestingly, partial restoration of the right horizontal plate and the right orbital process of the palatine bone can be seen in Dog-3 and Dog-4, respectively, but not the turbinate. These occurrences warranted further studies involving characterization of lysis and regrowth of the turbinate and adjacent bones affected by intranasal CTVT, which could be achieved via advanced sequential imaging such as CT.

All four dogs are free of clinical signs until now, with five cycles of vincristine sulfate. A positive response to therapy in this phenotype with various bony changes in all these cases indicates that staging would be more related to local tissue changes than the duration of treatment and outcome. Ojeda et al. (2018) also suggested similar results regarding the correlation of the staging system with treatment and outcome. Either radiotherapy or surgical excision can be an option, but not as monotherapy due to the poor remission (Raghunath et al., 2015).

Uncommonly, if it occurs, metastasis usually involves regional lymph nodes (Nemzek et al., 2015). In the present study, Dog-2 had unilateral left-sided submandibular lymphadenomegaly, but it was not tested for metastasis.

Vincristine sulfate is still an effective monotherapy to achieve full recovery upon the comparison of the previous and present primary nasal CTVT reports, although the number of cycles can vary, possibly depending on the expressed phenotype (Ganguly et al., 2013; Ojeda et al., 2018; Papazoglou et al., 2001; Veloso et al., 2018). In the past, resistance to this drug was reported in some cases, especially with the plasmacytoid phenotype. In such cases, alternatives such as doxorubicin or a combination of surgical strategies can be adopted to achieve complete remission (Papazoglou et al., 2001). Prognosis is not correlated to the staging system; it is quite variable and has been documented well to poor in previous studies (Ganguly et al., 2013; Ojeda et al., 2018; Tyagi et al., 2018). However, based on the present study results, the prognosis for vincristine sulfate usage in mixed phenotype cases seems good.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to this case study, authorship, and/or publication of this article.

DECLARATIONS

This work involved the use of non-experimental animals only (owned) and followed established internationally recognized high standards ('best practice') of individual veterinary clinical patient care. Therefore, ethical approval from a committee was not necessarily required.

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Relating Food Handlers' Knowledge, Attitude, and Self-Reported Practices on Food Safety and Hygiene to the Performance of Food Safety Assurance System: A Multiple Case Study in Government Hospital Kitchens

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ABSTRACT

Government hospital kitchens in Malaysia have been certified with Good Manufacturing Practices (GMP) and Hazard Analysis Critical Control Point (HACCP) to ensure that cooked food is clean and safe for consumption. However, the performances of the Food Safety Assurance System (FSAS)-certified government hospital kitchens have not been evaluated. Although researchers in Malaysia have assessed the knowledge, attitude, and self-reported practices (KAP) on food safety (FS) and hygiene among food handlers, they did not relate the influence of food handlers' KAP on the performance of the FSAS. The objective of the study was to relate food handlers' KAP on FS and hygiene to the FSAS performance in government hospital kitchens in Selangor and Kuala Lumpur, Malaysia. Four government hospital kitchens implementing different kinds of FSAS certification were evaluated. Critical sampling locations were identified, and samples were taken and examined for *Escherichia coli*, Total Yeast and Mould Count (TYMC), *Staphylococcus aureus*,

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Total Plate Count (TPC), and *Salmonella*. The average scores of knowledges on FS and hygiene for ≤ 30 years age group, ≤ 5 years in the employment group, and food handlers with tertiary education were the highest and significantly different compared with the other groups. The FS output of government hospital kitchens implementing stringent FSAS was better (score of 2–3) than kitchens implementing less stringent FSAS (score of 2). The multinomial logistic regression analysis showed that the correlations among the overall percentage scores of KAP and the FS output were not significant ($p > 0.05$). Therefore, it appeared that food handlers' KAP on FS and hygiene did not influence *Salmonella*, *E. coli*, and *S. aureus* levels and, therefore, the FSAS' performance.

Keywords: Food handlers, food safety assurance system, food safety output, government hospital kitchens

INTRODUCTION

Hospital food is an essential source of nutrition for inpatients (Yousif et al., 2013). More than 75% of the population depended on the in-house catering menus during hospitalization (Allison, 2003). Therefore, providing safe food for consumption is a significant responsibility, especially for the young, the elderly, and the immune-suppressed patients (Lund & O'Brien, 2011; Valero et al., 2016). Outbreaks of nosocomial foodborne illness can have severe implications, incurring additional medical costs to the healthcare setting and the risk of spreading the infection to other patients (Lund & O'Brien, 2011). Food is generally safe when free from microbial, chemical, and foreign contaminants (Lund & O'Brien, 2011). The Food Safety Assurance System (FSAS) is therefore enforced in food service establishments by the authorities to ensure food safety (FS) and quality and to avoid foodborne outbreaks (Jacxsens et al., 2009).

Furthermore, Food Hygiene Regulations 2009 have made it mandatory for food premises in Malaysia to implement a Food Safety Assurance Program (Laws of Malaysia., 2009). In addition, most safety and quality certification systems, such as Hazard Analysis Critical Control Point (HACCP) and ISO 22000 Food Safety Management System (FSMS), require an organization to establish a measurable FS objective to measure its performance (Standards Malaysia, 2007, 2012). FS objective specifies the maximum permissible level of a microbiological hazard in a food at the moment of consumption, and it provides the industry with quantitative targets to be met (van Schothorst et al., 2009). In addition, different authors used Microbial Assessment Scheme (MAS) to evaluate FSAS' performance in an organization, in which the selected microbiological parameters and conditions were used to establish FS objectives (Jacxsens et al., 2009; Oses et al., 2012).

Foodborne illness in food service has been associated with FS behavior and the practice of food handlers, where poor personal hygiene and improper holding time and temperature are primarily implicated (Abdul-Mutalib et al., 2015). Knowledge

of food hygiene practices is essential for the food handlers to have the right attitude to perform their duties and produce safe foods for consumption (Baluka et al., 2015). Researchers have used surveys to evaluate the knowledge, attitude, and self-reported practices (KAP) among hospital food handlers (Bou-Mitri et al., 2018; Norhaslinda et al., 2016). Food handlers are usually deemed to have good KAP if they score ≥ 80% of the total marks (Nyarugwe et al., 2018; Pacholewicz et al., 2016). Although different researchers in Malaysia have assessed the KAP among food handlers (Norhaslinda et al., 2016; Suhaila et al., 2020; Tan et al., 2013), they did not relate the influence of food handlers' KAP on the performance of the FSAS.

As mentioned earlier, the MAS has been carefully designed so that its microbiological results can serve as a numerical output in assessing the organization's FS objectives and, therefore, the performance of FSAS (Jacxsens et al., 2009). As a rule of thumb, low numbers of microorganisms and minor variations in the microbial counts indicate an effective FSAS. Researchers have effectively used MAS and KAP surveys to assess the influence of food handlers on the performance of FSAS in a variety of settings (Nyarugwe et al., 2018; Pacholewicz et al., 2016). For example, Nyarugwe et al. (2018) used MAS and KAP surveys, in addition to participatory observation, card-aided interviews, storytelling, and documentation analysis, to assess the performance of FSAS in the milk processing industry in Zimbabwe. Pacholewicz et al. (2016) used MAS and KAP surveys, in addition to observational study and documentation analysis, to assess the influence of food handlers' compliance with procedures of chicken abattoirs in the Netherlands. Government hospital kitchens in Malaysia have been certified with GMP and HACCP to ensure that cooked food is clean and safe for consumption (Ministry of Health, 2018). However, the performances of the FSAS-certified government hospital kitchens have not been evaluated. Therefore, the objective of the present work was to assess the influence of food handlers' KAP on FS and hygiene on the performance of the FSAS in selected government hospital kitchens around Selangor and Putrajaya, Malaysia.

MATERIALS AND METHODS

Characteristics of Government Hospital Kitchens

The present study was conducted in the kitchen of four government hospitals in Selangor and Kuala Lumpur, Malaysia, from 1st August until 31st October 2017. The following codes were given to the government hospital kitchens to disguise their identity: HK1, which is in Kuala Lumpur, and HK2, HK3, and HK4, located in Selangor. HK2 is Good Manufacturing Practices (GMP)-certified, whereas HK1, HK3, and HK4 are GMP- and HACCP-certified. Suhaila et al. (2020) reported using a questionnaire to assess the basic food safety and hygiene knowledge, attitude, and practice (KAP) of food handlers in these government hospital kitchens. A total of 140 food handlers participated in the reported work.

Microbial Assessment Scheme (MAS)

The MAS illustrated by Jacxsens et al. (2009) was used. It comprised of the following: (i) critical sampling locations, (ii) sampling frequencies, (iii) microbiological parameters and methods, (iv) sample preparation of final products, (v) air quality sampling, (vi) swab test's personal hygiene, (vii) food contact surfaces sampling, and (viii) tap water sampling.

Critical Sampling Locations (CSLs)

CSLs were chosen to concur with Jacxsens et al. (2009). The CSLs included final food products, cleanliness of preparation utensils (chopping board, working table, and serving trays), tap water, the air quality of serving and processing areas, and personal hygiene (pre-and post- washing hand) of the participating food handlers.

Sampling Frequencies

A visit was organized to each government hospital kitchen to collect samples at the chosen CSLs. Duplicate samples (n = 2) were collected each for the following: (i) final food products (chicken meals), (ii) tap water, (iii) food contact surfaces — chopping board, (iv) food contact surfaces — working table, (v) food contact surfaces — food tray, (vi) air quality, (vii) the hands of food handlers (pre-washing hand), and (viii) the hands of food handlers (post-washing hand). Sixty-four (64) samples (8 CSLs × 4 government hospital kitchens × 2 duplicates) were obtained.

Microbiological Parameters and Methods

The guidelines for the microbiological examination of ready-to-eat (RTE) foods (Food Standards Australia New Zealand [FSANZ], 2016) were used to choose microbiological parameters. Salmonella was identified as the FS indicator. *Escherichia coli* and *Staphylococcus aureus* were identified as the hygiene indicator. Furthermore, Total Yeast and Mould Count (TYMC) and Total Plate Count (TPC) were identified as the environmental and total microbiological qualities indicators, respectively. Plate Count Agar (PCA), Eosin Methylene Blue Agar (EMB), Xylose Lysine Deoxycholate (XLD) agar, and Buffered Peptone Water (BPW) beside Potato Dextrose Agar (PDA) were obtained from Oxoid (United Kingdom) and made corresponding to the guidelines provided by the producer. Petrifilm™ Staph Express Count Plate was obtained from 3M™ (USA).

Sample Preparation of Food Products

The International Commission on Microbiological Specifications for Foods (ICMSF) (2005) was used to enumerate TPC, *E. coli*, and *S. aureus* counts. Approximately 300 g of food samples were collected, and 25 g was mixed with Buffered Peptone Water (225 mL) in a sterilized stomacher bag, employing a stomacher for 2 min. The mixed samples were successively diluted with 1% peptone water equal to 10^{-6}. At every dilution factor, 0.1 mL of the liquid was transferred and applied uniformly

onto EMB, PCA, and Petrifilm™ Staph Express Count Plate test kits (USA). The detection of *Salmonella* was performed following International Organization for Standardization (ISO) 6579-1 (2017). The samples were pre-enriched in buffered peptone broth, and *Salmonella* was detected in XLD. Approximately 25–250 colonies were enumerated, utilizing a colony counter after incubation at 37 °C (18-24 h). As for the test kits, 15-150 colonies were counted. Then, the average colony count was determined and reported as $\log_{10}$ CFU/g. The isolates found were recognized as illustrated by the supplier of the media (Oxoid, United Kingdom; Biokar COMPASS®, France; and 3M™, USA). The experiments were conducted in duplicates.

Air Quality Sampling

The culture settling plating technique environment of serving and processing areas was examined (Salustiano et al., 2003). First, PDA plates were uncovered in the processing and serving areas for 15 min. Subsequently, the plates were covered after 15 min of air exposure and kept at 21 °C for 48-72 h. The resulting colonies in the range of 25-250 were calculated using a colony counter. Then, the average colony count was determined and reported as $\log_{10}$ CFU/m^3. Finally, the experiments were conducted in duplicates.

Swab Test's Hygiene

The swab test was executed before and after food handlers cleaned their hands, corresponding to ISO 18593 (2004). The sterilized swab was immersed in 1% peptone water and instantly swabbed in a region of about 25 cm^2. Subsequently, the head of the swab was lightly dipped in the peptone water and preserved in an icebox. Further, the samples were successively diluted, equal to 10^{-5} dilutions. Consequently, 0.1 mL of the sample at every dilution was pipetted and removed onto Petrifilm™ Staph Express Count Plate test kits (USA). The test kits were kept warm at 37 °C for 24-48 h. At the end of the incubation period, 15-150 colonies were calculated, utilizing a colony counter (Today's Instruments, Taiwan). The experiments were conducted in duplicates.

Food Contact Surfaces Sampling

The swab test was also conducted to sample the food contact surfaces after cleaning, as described in Swab Test's Hygiene sub-section above. The food-contact surfaces selected were the chopping board, working table, and serving tray. The swabbed stick was transferred to the laboratory using an icebox. The culture medium used was EMB to detect the occurrence of *E. coli*. The isolates attained were recognized as depicted by the media provided by the manufacturer. The experiments were conducted in duplicates.

Tap Water Sampling

Escherichia coli counts of the samples were completed, following Rosmawati et al. (2014). Serial dilutions equal to 10^{-6} were performed. Approximately 0.1 mL of sample was removed and spread evenly onto EMB plates at every dilution. The plates

were kept warm at 37 °C (8-24 h). Only 25-250 colonies were enumerated using a colony counter. Then, the colony count was determined and reported as $\log_{10}$ CFU/mL. The isolates obtained were identified as described by the supplier of the media (Oxoid, United Kingdom). The experiments were conducted in duplicates.

Microbiological Criteria and Interpretation

The microbial counts of chicken meals were evaluated in contrast to the guidelines for the microbiological examination of ready-to-eat (RTE) foods (FSANZ, 2016) and deemed unsafe for utilization if the count was higher than the allowable limit. According to FSANZ (2016), the permitted levels are *Salmonella* must not present in 25 g, *E. coli* < 3 CFU/g, *S. aureus* < 10^2 CFU/g, and TPC < 10^4 CFU/g. In addition, the enumerated microbial counts of food contact surfaces and hands were regarded as unacceptable when the count is equal to or higher than that present in the food samples (Oses et al., 2012). *Escherichia coli* in tap water must be absent in 100 mL (Laws of Malaysia., 1985). The maximum value for TYMC is suggested by Sveum et al. (1992) and must not surpass 90 CFU/m^3.

According to Jacxsens et al. (2009), the microbiological safety level can be categorized as low, medium, and high. An FS level of 1 to 3 was utilized at different CSLs for each microbiological parameter (Jacxsens et al., 2009). More specifically, levels 1 indicate "poor", level 2 indicates "moderate", and level 3 indicates "good" FS levels. Level 1 specifies that improvements are required on numerous control activities when legal conditions or guidelines are surpassed. Level 2 specifies that improvements are required on one control activity, especially when legal conditions or guidelines are surpassed. Level 3 specifies that no improvement is required; legal conditions or guidelines are followed. The total of the microbiological safety levels for each parameter might attain a maximum of 15 (5 × 3). The score of 1, i.e., "poor", was designated when the total of the levels was 5 to 7; a total of 8 to 9 ensued in a score of 1–2, i.e., "poor to moderate", a total of 10 to 11 ensued in a score of 2, i.e., "moderate", a total of 12 to 13 ensued in a score of 2–3, i.e., "moderate to good", and a total of 14 to 15 ensued in a score of 3, i.e., "good".

Statistical Analysis

The obtained data were processed and analyzed using Statistical Package for the Social Sciences (SPSS) (version 16). One-way analysis of variance (ANOVA) was used to assess the effects of the demographic variables (i.e., age group and length of employment) on the average score of KAP of food handlers. In addition, an independent-samples *t*-test was used to assess the effects of the demographic variables (i.e., gender and educational background) on the average score of KAP of food handlers. Tukey's multiple comparisons test was used to determine the significance of the differences in the average scores. Moreover, multinomial logistic

regression, which allows for a dependent variable with more than two categories, was used to determine the correlation between the overall percentage scores of KAP and the FS output of MAS. As mentioned, an arbitrary scale was used to interpret the overall percentage scores of KAP into good (> 80%), moderate (51% – 79%), or poor (< 50%) (Nyarugwe et al., 2018; Pacholewicz et al., 2016). These arbitrary scales were used as independent variables. In contrast, the FS outputs have five categories: 1 (poor risk), 1–2 (poor to moderate level), 2 (moderate-risk), 2–3 (moderate to a good level), and 3 (good level), were used as the dependent variable. A p-value ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

The present work assessed the relationship of food handlers' KAP on FS and hygiene on the microbiological performance of the foods served to the patients in government hospital kitchens. Table 1 shows the effect of demographic variables on the average score of KAP on FS and hygiene among food handlers. There was no significant difference ($p > 0.05$) in the average knowledge score on FS and hygiene between males and females. However, there was a significant difference ($p \leq 0.05$) among age groups, length of employment, and educational background. The tertiary education group's average FS knowledge and hygiene scores were at 41.5%, which were the highest ($p \leq 0.05$) and significantly different than other groups. In contrast, the ≤ 30 years age group is at 40.7%, and the ≤ 5 years length of employment group is at 41.9%. There was no significant difference ($p > 0.05$) in the average score of attitudes on FS and hygiene for the following demographic variables: gender, age group, length of employment, and educational background.

The average scores of knowledges on FS and hygiene for ≤ 30 years age group,

Table 1
*Effect of demographic variables on the average score (%) of knowledge on food safety and hygiene among food handlers (*n = *140)*

Demographic variables	Levels (*n*)	Average score % (SD)	df	F	*p*
Gender	Male (*n* = 26)	39.4 (3.61)	138	2.12	0.374
	Female (*n* = 114)	38.4 (3.32)			
Age group	≤ 30 years (*n* = 42)	40.7[a] (3.77)			
	31 - 40 years (*n* = 56)	38.9[a] (5.55)	138	9.59	0.000*
	≥ 41 years (*n* = 42)	36.1[b] (4.90)			
Length of employment	≤ 5 years (*n* = 30)	41.9[a] (3.21)			
	6 - 10 years (*n* = 50)	38.7[b] (4.82)	138	11.9	0.000*
	≥ 11 years (*n* = 60)	36.8[b] (5.18)			
Educational background	Tertiary (*n* = 51)	41.5[a] (3.77)	138	6.08	0.000*
	Secondary and below (*n* = 89)	36.9[b] (4.96)			

*$p \leq 0.05$
[a] Values = The average (standard deviation)
[a-b]Values within the same column with different letters are significantly different

≤ 5 years in the employment group, and food handlers with tertiary education were the highest and significantly different ($p \leq 0.05$) from the other groups (Table 1). The effective management of the FSAS can be improved by applying precautionary approaches and providing continuous training to food handlers on food hygiene and FS (Akabanda et al., 2017). Continuous training needs to be given, especially on the aspect of time and temperature controls, to food handlers with an educational background of secondary and below, in which they are typically aged ≥ 41 years old and have been employed for ≥ 11 years. Food handlers that belong to these identified groups shall be the target group for future food handler refresher training. According to Pacholewicz et al. (2016), consistent FS and hygiene compliance (brought about by continuous training) will improve product safety performance. Therefore, food handlers must be continuously motivated, guided, and educated to ensure FS and hygiene.

A similar trend was also observed for self-reported practices on FS and hygiene except for educational backgrounds. However, the average score of self-reported practices on FS and hygiene for the group with tertiary education was higher and significantly different ($p \leq 0.05$) as compared with the secondary and below a group of employees (data not shown).

Table 2 shows the FS outputs of the government hospital kitchens according to the Microbiological Assessment Scheme (MAS). It was observed that the score of FS output for HK1, HK3, and HK4 was "moderate to good", i.e., a score of 2–3, and "moderate", i.e., a score of 2 for HK2. Furthermore, multinomial logistic regression analysis showed that the correlations among the overall percentage scores of KAP and the FS output of MAS were not significant ($p > 0.05$) (data not shown).

It was found that the FS output of government hospital kitchens (HK1, HK3, and HK4) implementing stringent FSAS (GMP- and HACCP-certified) achieved a "moderate to good" level (score of 2-3). On the other hand, HK2, which implements less stringent FSAS (GMP-certified), recorded a "moderate" level (score of 2) (Table 2). The microbiological criteria selected were the hospitals' FS objectives. *Escherichia coli*, *S. aureus*, and TPC were not in chicken meals from HK1, HK3, and HK4. However, they were present at levels above the allowable levels in samples from HK2. The microbiological safety of government hospital kitchens implementing stringent FSAS was better than hospital kitchens implementing less stringent FSAS. Our findings align with the results of Nyarugwe et al. (2018), who evaluated the performance of microbiological safety of three Zimbabwean dairy companies with different levels of implemented FSAS. They reported that companies certified with HACCP had a better microbiological safety performance than those not HACCP-certified. Implementation of HACCP principles require the development of a HACCP plan. HACCP plan defines the procedures for maintaining control of

Table 2
The number of samples exceeding the limiting criteria for Staphylococcus aureus, Escherichia coli, Salmonella, *Total Yeast and Mould Count, and Total Plate Count over different critical sampling locations (CSLs), the food safety (FS) Levels attributed for all microbiological parameters, and the FS outputs at different government hospital kitchens*

CSL[1]	HK1[2]	HK2[3]	HK3[4]	HK4[5]
Escherichia coli				
Food product — Chicken meals (n^6 = 2)	ND[7]	2	ND	ND
Equipment: Chopping board (n = 2)	2	ND	ND	1
: Working table (n = 2)	ND	ND	2	ND
: Food tray (n =2)	ND	ND	2	ND
Tap water (n = 2)	ND	ND	ND	ND
FS Level[9]	2	2	1	2
Staphylococcus aureus				
Food product — Chicken meals (n = 2)	ND	2	ND	ND
Personnel hygiene: Before (n = 2)	2	2	1	2
: After (n = 2)	2	2	ND	2
FS level	1	1	2	1
Salmonella				
Food product — Chicken meals (n = 2)	ND	ND	ND	ND
FS level	3	3	3	3
Total Plate Count				
Food product — Chicken meals (n = 2)	ND	2	ND	ND
FS level	3	1	3	3
Total Yeast and Mould Count				
Air quality in processing and serving area (n = 2)	0[8]	0	0	0
FS level[9]	3	3	3	3
FS output[10]	(2-3)	(2)	(2-3)	(2-3)

[1]CSL = Critical sampling location
[2]Government hospital kitchen certified with Good Manufacturing Practices (GMP) and Hazard Analysis Critical Control Point Principles (HACCP)
[3]Government hospital certified with Good Manufacturing Practices (GMP)
[4]Government hospital certified with Good Manufacturing Practices (GMP) and Hazard Analysis Critical Control Point Principles (HACCP)
[5]Government hospital certified with Good Manufacturing Practices (GMP) and Hazard Analysis Critical Control Point Principles (HACCP)
[6]Total number of samples per CSL at different hospital kitchens
[7]Below detection limit (ND)
[8]Counts were found, but below the criteria
[9]Level 1 — Low result (legal criteria or guidelines are exceeded, improvements need to be made on multiple control activities of the FSAS). Level 2 — Medium result (legal criteria or guidelines are exceeded, improvements need to be made on a single control activity of FSAS). Level 3 — Good result (legal criteria or guidelines are respected, no improvements are needed — current level of FSAS is high enough to cover this hazard)
[10]The score of 1, i.e., "poor", was designated when the total of the levels was 5 to 7; a total of 8 to 9 ensued in a score of 1–2, i.e., "poor to moderate", a total of 10 to 11 ensued in a score of 2, i.e., "moderate", a total of 12 to 13 ensued in a score of 2–3, i.e., "moderate to good", and a total of 14 to 15 ensued in a score of 3, i.e., "good"

potentially hazardous food at the critical control points of food preparation or processing to ensure that the measurable FS objectives can be met (Standards Malaysia, 2007). On the other hand, GMP set regulations, codes, and guidelines that control the operational conditions within a food establishment, allowing for safe food production. GMP is also one of the pre-requisite programs for establishing the HACCP System (Standards Malaysia, 2013). Kokkinakis et al. (2008) showed that implementing stringent FSAS (GMP- and HACCP-certified) through the application of good practices (GMP and good hygiene practices) in addition to the HACCP plan can improve product safety. Due to the HACCP plan being maintained and periodically verified in government hospital kitchens implementing stringent FSAS (GMP- and HACCP-certified), their microbiological safety performance was better than in kitchens implementing less stringent FSAS (GMP-certified).

Nevertheless, none of the government hospital kitchens could accomplish a good level of FS performance, ultimately their FS objectives. The microbiological results attained at the CSLs in the hospital kitchens exceeded the permitted levels. The load of *E. coli* on the equipment and utensils above the allowable levels in several government hospital kitchens was in agreement with Yousif et al. (2013). They also reported that the equipment surface swabs in a hospital kitchen in Egypt were infected with *E. coli* above the allowable level. It might be due to poor hygiene and water disinfectants. Therefore, the focus on the cleaning procedure of the food contact surfaces should be given to minimize the microbial risk of contamination (De Souza, 2003). *Escherichia coli* was detected on the surface of the equipment for HK1 and HK4 (chopping board) as well as HK3 (working table and food tray) but not detected in the chicken meals of these government hospital kitchens (Table 2). The heating process appears sufficient to inactivate *E. coli* in the final product. These results are similar to those described by Fernandes et al. (2017), who showed that *E. coli* was detected in 6.7% of the surface samples but not detected in the final product. Cooking remains a primary means of eliminating pathogens with heat to achieve a specific lethality, and it continues to play a significant role in preventing future outbreaks in meat products (Murphy et al., 2004). On the contrary, *E. coli* was not detected on the surface of the equipment for HK2, but its levels were above the limit in the chicken meals in this hospital kitchen. In addition, as mentioned, *S. aureus* and TPC were detected in the chicken meals for HK2 at levels above the permitted limits (Table 2). HK2 is certified with GMP only and implements less stringent FSAS compared with HK1, HK3, and HK4. It is possible that the application of good practices (GMP and good hygiene practices) without implementation of the HACCP plan is insufficient to ensure the FS objectives can be met.

It was also found that *S. aureus* was present in the chicken meals above the permitted limit of 10^2 CFU/g and on the

food handlers' hands is equal to or higher than that present in the chicken meals. These findings are consistent with Dablool and Ghamdi (2011), who found that the hands of food handlers were contaminated with *S. aureus*, which is a major human pathogen capable of causing a wide range of infections, including food poisoning caused by the enterotoxin produced by the pathogen (De Sousa, 2008). Moreover, Dablool and Ghamdi (2011) highlighted that staphylococcal food poisoning (SFP) outbreaks in the retail industry are primarily due to the food handler's inappropriate food handling practices and poor personal hygiene. The literature mostly reported the SFP outbreak in restaurants, especially in Brazil (Carmo et al., 2003), Italy (Ercoli et al., 2017), and less in hospital kitchen settings. The incidents of SFP outbreaks in Malaysia are mainly reported in school canteens (Abdullah & Ismail, 2021; Lekhraj, 1983) and during the mass gathering (Rajakrishnan et al., 2022).

In addition, *E. coli* and *S. aureus* were selected as the hygiene indicators, and their results in all the government hospital kitchens were above acceptable levels. It was reported that food handlers that are working in government hospital kitchens have "good" knowledge as well as a "moderate to good" attitude and self-reported practices for hand and personal hygiene (Suhaila et al., 2020). However, it did not influence the levels of *E. coli* and *S. aureus* and, therefore, the performance of FSAS in the government hospital kitchens. Multinomial logistic regression analysis was performed to confirm the observation and found that the correlations among the overall percentage scores of KAP and the FS output of MAS were not significant ($p > 0.05$). Lee et al. (2017) assessed the KAP of FS and hygiene and microbiological hand hygiene of food handlers in university canteens. They reported similar findings in which "moderate" performance on FS knowledge was not reflected in the microbiological hygiene assessment of hands. Adesokan et al. (2015) showed that FS training is associated with improved knowledge and practices among food handlers. However, other factors besides knowledge, attitude, and practices in terms of FS and hygiene might affect the FS output, such as enabling conditions and actual behavior, as demonstrated by other researchers (Nyarugwe et al., 2018; Pacholewicz et al., 2016).

Suhaila et al. (2020) reported that the KAP of food handlers on time and temperature controls varied between "poor" and "moderate". They also reported that the KAP of food handlers on cross-contamination varied from "poor" to "moderate" and "good". However, *Salmonella*, selected as the FS indicator, was not detected in the food product in the present work. Therefore, it can be deduced that "poor" to "moderate" KAP of food handlers on time and temperature controls, as well as "poor" to "moderate" and "good" KAP of food handlers on cross-contamination, did not influence the levels of *Salmonella*. Our results, however, contradicted Lee et al. (2017). They reported that "good" self-

reported practices were not reflected in the microbiological assessment of food handlers' hands, in which *Salmonella* was detected in 48% of the food handlers' hands. Since the FSAS was enforced in all the government hospital kitchens tested in the present work, it was likely that quality control and assurance systems were already in place to control pathogens such as *Salmonella*, unlike the study by Lee et al. (2017), which was conducted in uncertified university canteens. Furthermore, Kokkinakis et al. (2008) have demonstrated the positive effects that HACCP had in an ice cream factory, which was reflected in the improved microbiological quality of the final products.

CONCLUSION

The present study showed that the FS outputs of government hospital kitchens implementing stringent FSAS demonstrated a better performance ("moderate to good") than the one with less stringent FSAS (only "moderate"). Nevertheless, none of the government hospital kitchens could accomplish a good level of FS performance; ultimately, their FS objectives. The multinomial logistic regression analysis showed that the correlations among the overall percentage scores of KAP and the FS output were not significant ($p > 0.05$). Therefore, it appeared that food handlers' KAP on FS and hygiene did not influence the FS outputs and, therefore, the performance of the FSAS. The present study identified the target group with the following specific characteristics: food handlers with an educational background of secondary and below, aged ≥ 41 years old, and have been employed for ≥ 11 years. This target group requires continuous training to improve the attitude and self-reported practices on FS and hygiene practices to ensure a good level of FS performance and, therefore, the FS objectives are achieved. The present study collected duplicate samples ($n = 2$) at the chosen CSLs in each government hospital kitchen. The sampling numbers could be increased in future research to increase data confidence.

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Genetic Diversity of *Exobasidium vexans*, the Causal Agent of Blister Blight on Tea in Pagilaran, Central Java, Indonesia Using PCR-RAPD

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ABSTRACT

Indonesia is one of the ten largest tea-producing countries in the world, with a plantation area of 104,420 hectares and a production of 139,285 thousand tons in 2018. Blister blight can cause massive crop losses across tea-growing regions of Asia, particularly in India, Sri Lanka, Indonesia, and Japan. The infection causes a 40% yield loss. The study aimed to determine the genetic diversity in *Exobasidium vexans* that cause blister blight based on polymerase chain reaction-random amplified polymorphic DNA (PCR-RAPD). Sampling was conducted at Pagilaran, a tea plantation located in Central Java, Indonesia, with sampling based on altitude, Andongsili (>1,000 meters above sea level [masl]), Kayulandak (±1,000 masl), and Pagilaran (<1,000 masl) with clones TRI 2024, TRI 2025, Gambung 3, Gambung 7, Gambung 9, and Pagilaran 15. This study used the PCR method using internal transcribed spacers (ITS) 1F and ITS 4 primers. Four primers used in PCR-RAPD were OPA-02, OPA-03, OPA-05, and OPB-17. The characteristics of *E. vexans* observed were ellipse-shaped basidiospore, hyaline, unicellular with one septate, formed at the tip of the sterigma with hyaline and elliptical shapes, with a range size of 7–15.5 µm x 2.3–4.5 µm. PCR-RAPD method was able to show the diversity of *E. vexans* samples between clones, in which three clusters were formed at a coefficient of 0.63. Cluster I consisted of TRI 2024 Andongsili and PGL 15 Pagilaran; Cluster II consisted of TRI 2025 Andongsili and Gambung 3 Andongsili; Cluster III consisted of Gambung 7 Andongsili, Gambung 7 Kayulandak, and Gambung 9 Andongsili.

Keywords: Altitude, blister blight, *Exobasidium vexans*, genetic diversity

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INTRODUCTION

Global tea production increased with an average annual growth of 4.7% over the last decade, reaching 5.98 million tons in 2018. Continued global volume growth generated by China's tea production has nearly doubled since 2009, reaching 2.616 million tons in 2018, which is 44.4% of the world's tea. This massive expansion meets unprecedented domestic demand (Dufrene, 2020). Indonesia is one of the ten largest tea-producing countries globally, with a plantation area of 104,420 ha and a production of 139,285 thousand tons in 2018 (Sub Directorate of Estate Crops Statistics, 2019). Climate change has a major impact on the growth and development of tea plants. According to Ochieng et al. (2016), tea is a plant easily affected by climate change. Significant temperature changes and extreme weather pose a threat to the tea production system. Blister blight (*Exobasidium vexans* Massee) is a major disease in various countries. This disease becomes more prevalent during the rainy season and may become more severe in regions where the monsoon season lasts longer. Microorganisms have also been demonstrated to impact tea quality, with pathogen infection lowering the levels of some secondary metabolites (Ahmed et al., 2018). *Exobasidium vexans* is a biotroph pathogenic fungus that infects shoots. The infection develops by forming a shiny concave lesion on the upper surface and a white powdered lower surface. As the disease progresses, the leaves are malformed and roll (Ahuja et al., 2013). Blister blight can cause massive crop losses across Asia's tea-growing regions, particularly in India, Sri Lanka, Indonesia, and Japan. The infection causes a 40% yield loss (Basu et al., 2010).

Disease management recommendation for blister blight is the application of copper (Cu)-based fungicides directly to the leaves before infection occurs. Nevertheless, the maximum residue level of pesticides causes the tea to be less desirable to consumers (Barooahi et al., 2002). A viable and effective option for the management of blister blight can be made through the genetic improvement of tea resistance (Karunarathna et al., 2020). Molecular marker technology is currently used in tea genetic diversity studies to acquire population structures and uncover the history of domestication (Meegahakumbura et al., 2018). Molecular marker technology provides a rapid set of polymorphic DNA-based markers with advantages of a wide range of applications in plant genetics and breeding (Lee et al., 2019).

Morphological observations of *E. vexans* spores to detect and distinguish the sequences can help compare the variability and similarity of *E. vexans* in different tea-growing areas. The molecular characteristics of *E. vexans* needed to be documented for further characterization studies. Information on the genetic and morphological diversity of *E. vexans* is necessary to support the development of effective control for blister blight (Abeysinghe et al., 2015). Limited information about genetic diversity in the *E. vexans* causes the need for molecular

study, especially using RAPD. This technique is an easy, simple, and economical technique for the population's genetic diversity analysis and does not require any sequence information (Joshi et al., 2009). RAPD analysis is performed to determine intraspecific variability in a species. Similarity measurements using unweighted pair group with arithmetic mean (UPGMA) program in numerical taxonomy system (NTsys) (version 2.1) and continued cluster analysis generally reflect trends between genotypes. RAPD markers are dominant, unable to distinguish amplified DNA from heterophile or homozygous loci. The quality and concentration of DNA templates, PCR component concentrations, and PCR cycle conditions affect results (Nandani & Thakur, 2014). The study aimed to determine the genetic diversity in *E. vexans* that cause blister blight based on PCR-RAPD.

MATERIAL AND METHOD

Sampling and Specimen Collection

Sampling was done by exploring the symptomatic leaves with symptom criteria in stages 2 (blister formation stage) and 3 (incipient stage of sporulation). The samples were then collected in an envelope. The basidiospores were scraped using toothpicks and then put into 1.5 ml Eppendorf tubes containing 1 ml 6% sucrose solution. Each Eppendorf tube was filled with basidiospores from 20–30 leaves with blister blight symptoms. The samples were stored at a temperature of -20 °C.

Sampling was conducted in the PT Pagilaran tea plantation, Central Java, Indonesia. The sampling site was divided by altitude (high, middle, and low), which was grouped into three criteria: high >1,000 masl, middle ±1,000 masl, and low <1,000 masl. Selected blocks include Andongsili (high), Kayulandak (middle), and Pagilaran (low). At each site, samples of blister blight were collected from TRI 2024, TRI 2025, Gambung 3, Gambung 7, Gambung 9, PGL 6, PGL 11, and PGL 15 clones.

Morphological Character

The macroscopic characterization in this study was determined by observation with the naked eye, and the symptoms of blister blight were documented at the plantation. The symptomatic tea leaves obtained from each clone were then compared to other clones. For microscopic observation, each stored sample in a 6% sucrose solution was dripped on the object-glass, added with lactophenol cotton blue solution, and then covered with a glass cover. The single spore was observed for each replication at three groups of altitude locations using 40x magnification and measured using Image Raster (version 4.0) (Miconos, Indonesia).

DNA Extraction and Amplification

DNA extraction was done using the Aboul-Maaty et al. (2019) protocol with modifications adjusted to laboratory conditions. DNA extraction of *E. vexans* was carried out using the 3% (w/v) cetyltrimethylammonium bromide (CTAB) method. The 3% (w/v) CTAB (Vivantis, Malaysia) heated at 65 °C and added with 0.3% (v/v) beta-mercaptoethanol (Merck,

Germany). The 50 µl 1× TE buffer (Tris-ethylenediaminetetraacetic acid) (Vivantis, Malaysia) was incubated with 3% (w/v) CTAB. A total of 50 mg of the sample was grounded and then put into a 1.5 ml Eppendorf tube. The sample was then added with 800 ml of 3% (w/v) CTAB that had been reheated. The mixture was incubated in a water bath at 60-65 °C for 1 hour (the sample was turned around every 20 minutes). Chloroform isoamyl alcohol 24:1 (v/v) (Merck, Germany) was added to the sample and shaken until homogeneous. The sample was centrifuged at 10,000 x *g* for 15 minutes, and the top solution was transferred into the new Eppendorf. The 6 M sodium chloride (NaCl) (Vivantis, Malaysia) was added to as many as 0.5 volumes of the supernatant obtained and then shaken until homogeneous. A much of 0.1 M potassium acetate (Merck, Germany) and 500 µl cold isopropanol (Merck, Germany) was added, then shaken until homogeneous. The supernatant was then stored at -20 °C for 30 minutes. The supernatant was centrifuged at 10,000 x *g* for 5 minutes, and the supernatant was discarded. The pellets were washed with 500 µl 70% ethanol and then shaken until homogeneous. The samples were centrifuged again at 10,000 x *g* for 5 minutes. The ethanol was discarded, and the pellets were dried for 15 minutes. The Eppendorf tubes containing pellets were incubated for 1-2 hours and then added with TE buffer. The DNA was stored at -20 °C.

The PCR components consist of 5 µl MyTaq™ HS Red Mix Bioline (Meridian Bioscience, USA), 3 µl ddH_2O, 0.5 µl forward and reverse primers, and 1 µl DNA templates. In this study, the ITS primary pair was ITS-1F and ITS-4, with a target size of about 600 bp (Mohktar & Nagao, 2019). The PCR program was as follows: an initial 3-minute pre-denaturation at 95 °C and 35 cycles of 30 seconds at 95 °C (denaturation), 30 seconds at 45 °C (annealing), and 1 minute at 72 °C (extension), followed by a final 5-minute extension at 72 °C. The amplicons and a 100 bp marker were included in the agarose well and a volume of 1-2 µl each. Next, the agarose gel was run in electrophoresis using Bio-Rad DNA Electrophoresis Cell (USA) at 70 V for 50 minutes with a 1x Tris-borate-EDTA (TBE) (Vivantis Technologies, Malaysia). Next, the 1% agarose gel was soaked in ethidium bromide (EtBr) for 15 minutes for DNA staining, then washed by soaking the gel in aqua dest for 5 minutes. Finally, the visualization was done on the UV transilluminator (Bio-Rad, USA).

PCR-RAPD

The primers used in RAPD analysis to determine the genetic diversity of *E. vexans* (Table 1) were OPA-02, OPA-06 (Joshi et al., 2009), OPB-17, and OPA-03 (Abeysinghe et al., 2015). The RAPD component consists of 7.5 µl MyTaq™ HS Red Mix, 5.7 µl ddH_2O, 1.5 µl DNA, and 0.3 µl primer. The PCR-RAPD program was as follows: an initial 3-minute pre-denaturation at 95 °C and 40 cycles of 30 seconds at 95 °C (denaturation), 30 seconds at 45 °C (annealing), and 1 minute at 72 °C (extension), followed by a final 5-minute extension at 72 °C.

Table 1
RAPD primers sequence

No.	Primer	Sequences	GC content (%)	Tm (°C)	Annealing (°C)
1.	OPA-02	5'- TGC CGA GCT G-3'	70	40.7	45
2.	OPA-03	5'- AGT CAG CCA C -3'	60	34.3	45
3.	OPA-06	5'- AAT CGG GCT G -3'	60	35.1	45
4.	OPB-17	5'- AGG GAA CGA G -3'	60	33.1	45

Note. GC content = Guanine-cytosine content; Tm = Melting temperature

The amplicons and a 100 bp marker were included in the agarose well with a volume of 1-2 µl. The agarose gel was then run-in electrophoresis using Bio-Rad DNA Electrophoresis Cell (USA) at 70 V for 50 minutes with a 1x TBE. The agarose gel was soaked in EtBr for 15 minutes for DNA staining, then washed by soaking the gel in aqua dest for 5 minutes. The visualization was done on the UV transilluminator (Bio-Rad, USA).

Data Analysis

The results of ITS 1F and ITS 4 gene sequences were compared to sequences obtained from National Center for Biotechnology (NCBI)-GenBank by conducting a nucleotide basic local alignment search tool (BLASTn) (https:/blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were generated by sorting the downloaded references region and analyzed by neighbor-joining at 1,000 bootstraps using Molecular Evolutionary Genetics Analysis (MEGA) (version 11). Each RAPD band was considered one putative locus. Only the locus showing a clear band was included in the scoring: band present (1) and no band (0). The matrix binaries phenotype RAPD was then compiled for individual cluster analysis using the UPGMA program in NTsys (version 2.1). Principal Coordinate Analysis (PCoA) was analyzed using paleontological statistics (PAST) software (version 4.03) based on the RAPD data. Similarity analysis based on FASTA DNA sequence data generated pairwise matrix similarity using Sequence Demarcation Tool (SDT) (version 1.2).

RESULT AND DISCUSSION

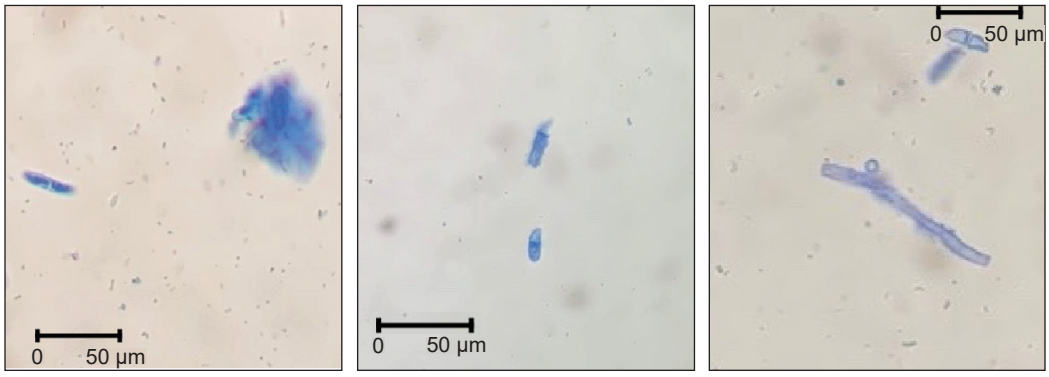
Morphological Character

Tea blister blight is indicated by the presence of white convex blister lesions on the lower surface of leaves, forming the typical symptom of blister blight. The advanced infection will cause necrotic symptoms. High temperature and humidity conditions support the germination of *E. vexans* spores. Symptomatic tea leaves taken from three different altitudes (Figure 1) showed no symptoms differences at different heights. The higher locations are foggy, and this condition will cause an increase in air humidity. High humidity and lack of sunlight are ideal conditions for the development of blister blight (Rezamela et al., 2016).

Based on Figure 2, the characteristics of *E. vexans* basidiospore were ellipses-shaped, hyaline, unicellular, and one septate. Spores

(a) (b) (c)

Figure 1. Macroscopic symptoms of blister blight: (a) TRI-2024 Andongsili, (b) Gambung 7 Kayulandak, and (c) PGL 15 Pagilaran, respectively



(a) (b) (c)

Figure 2. Microscopic observation of *Exobasidium vexans* spores: (a) TRI-2024 Andongsili, (b) Gambung 7 Kayulandak, and (c) PGL 15 Pagilaran, respectively

E. vexans are 15 x 5.5 µm. Basidiospores of *E. vexans* are formed at the top of the sterigma with hyaline and elliptical shapes measuring 7-15.5 µm x 2.3-4.5 µm. In the latest study by Chaliha and Kalita (2020), the germination of basidiospores can be performed *in vitro* on the agar surface after 4 hours post-incubation. After 8 hours post-incubation, hyphae formation differentiates into branches forming complex hyphae.

PCR Amplification

The use of pathogen nucleotide sequences was widely developed in line with PCR technique development as it has advantages of convenient use, speed, specificity, and sensitivity. Molecular technology is important for obligate pathogens that are difficult to grow in artificial media. Analysis of DNA sequences can be utilized to understand the evolution of pathogens and epidemics over time. PCR can be used as a genetic identifier for pathogens. Nilsson et al. (2014) explain that species identification is often difficult because fungi have complex life cycles and a large and diverse collection of eukaryotes. They also mentioned that DNA sequence is an important tool in identifying fungal plant pathogens by internal transcribed spacers (ITS). Its region is widely used to date for the amplification of fungal DNA. The primer pair ITS-1F and ITS-4 used in this study can amplify Ascomycota and Basidiomycota. *Exobasidium vexans* is a species categorized

into the Basidiomycota phylum. Mohktar and Nagao (2019) also used this primer pair for DNA amplification of *E. vexans*. Based on sequencing results, the species *E. vexans* is identified at 612-614 bp (Figure 3 and Table 2).

The phylogenetic tree (Figure 4) was constructed based on the sequence data of *E. vexans* in this study and other *Exobasidium* species in Genbank. Sequences are arranged using the maximum likelihood tree (MLE) on MEGA 11. Based on the data above, the sequence of seven samples used has a 100% similarity with *E. vexans* isolate MC32016 with accession number MG827276.1. The phylogenetic tree was divided into two clades based on the host group *E. vexans*, in this study based on the *Camellia* clade.

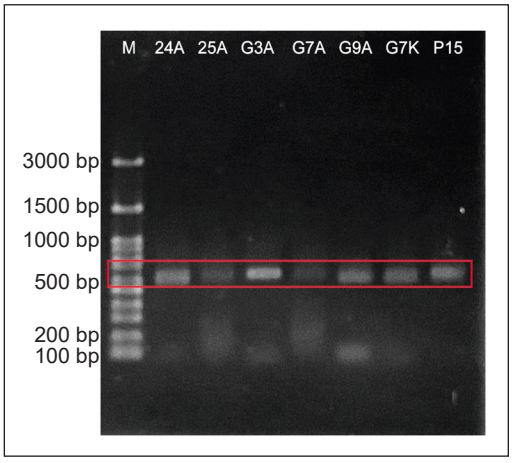


Figure 3. PCR amplification based on ITS sequences: 24A (TRI-2024 Andongsili), 25A (TRI-2025 Andongsili), G3A (Gambung 3 Andongsili), G7A (Gambung 7 Andongsili), G9A (Gambung 9 Andongsili), G7K (Gambung 7 Kayulandak), and P15 (PGL 15 Pagilaran), respectively

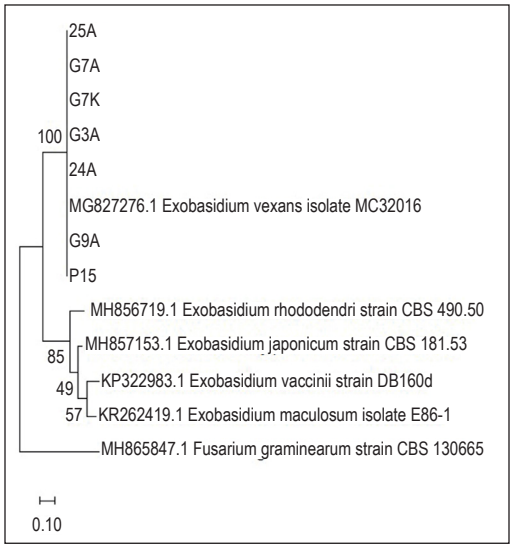


Figure 4. Phylogenetic tree of *Exobasidium vexans* based on the maximum likelihood of ITS sequence analysis

Table 2
Visualization of DNA sequence

Species	Isolate code	Amplification (bp)	Origin
Exobasidium vexans	24A	612	Andongsili
Exobasidium vexans	25A	613	Andongsili
Exobasidium vexans	G3A	612	Andongsili
Exobasidium vexans	G7A	612	Andongsili
Exobasidium vexans	G9A	614	Andongsili
Exobasidium vexans	G7K	612	Kayulandak
Exobasidium vexans	P15	613	Pagilaran

Note. 24A = TRI-2024 Andongsili; 25A = TRI-2025 Andongsili; G3A = Gambung 3 Andongsili; G7A = Gambung 7 Andongsili; G9A = Gambung 9 Andongsili; G7K = Gambung 7 Kayulandak; and P15 = PGL 15 Pagilaran

The separation of clade *E. vexans* with other species (*rhododendri*, *japonicum*, *vaccini*, *and maculosum*) is supported by an 85% bootstrap maximum likelihood value.

Molecular identification of *E. vexans* faces a major challenge where only a few ITS region sequences are stored in NCBI. Chaliha and Kalita (2020) said that this challenge encourages the development of specific molecular barcodes to identify *E. vexans*. In addition, the other challenge is the extraction of biotroph fungi to avoid the presence of secondary pathogens that can cause DNA contamination.

Based on the matrix data (Figure 5), the seven samples in the study had high similarity with *E. vexans* MC32016 isolate shown in red. It also confirmed that the *E. vexans* isolates in the study had a distance similarity with other *Exobasidium* sequences shown in blue. Matrix pairwise similarity confirms the similarity of *E. vexans* samples closed to *E. vexans* MC32016 isolate that corresponds with the phylogenetic tree in the study.

PCR-RAPD

The selection of four primers (Figure 6) showed that all of them produced polymorphic bands. These primers produce a clear, relatively stable, and easy-to-read polymorphic band. Primer selection for RAPD analysis determines the polymorphic bands' results as each primer

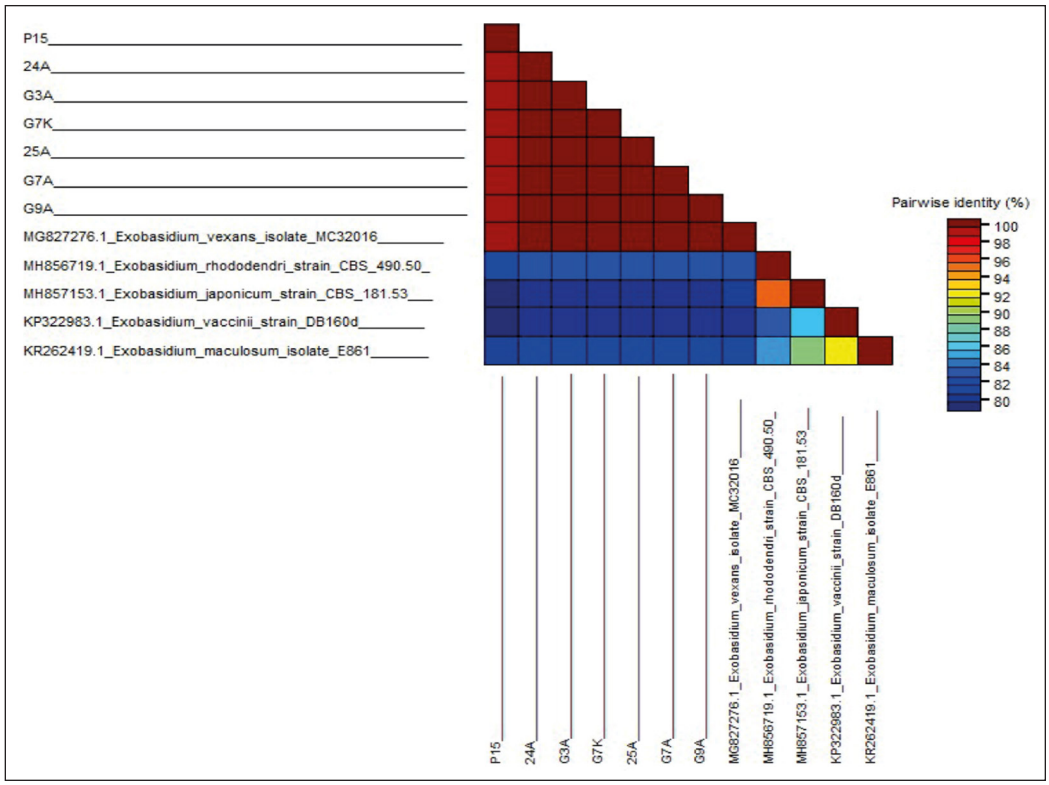


Figure 5. Pairwise similarity DNA of *Exobasidium*

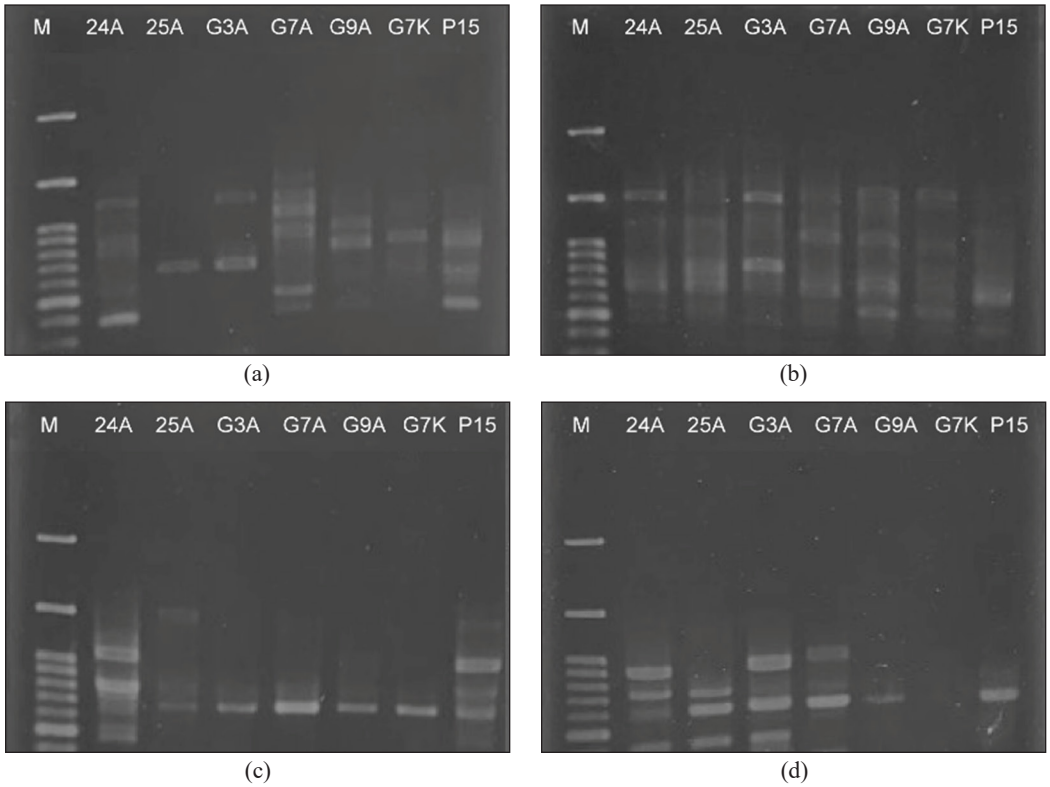


Figure 6. Visualization of PCR-RAPD results on 2% agarose gel, (a) OPA-02, (b) OPA-04, (c) OPA-06, and (d) OPB-17

has its attachment site. This feature results in polymorphic DNA produced differently, both the size and the number of DNA bands. The intensity of the amplified DNA bands is influenced by the purity and concentration of the DNA template (Gusmiaty et al., 2016).

The Pearson correlation test (Table 3) was used to determine the relationship between polymorphic information content (PIC) with effective multiplex ratio (EMR), PCR with marker index (MI), and EMR with MI. There is a positive correlation between PIC and EMR ($r = 0.935$; $p = 0.002 < 0.05$. A positive correlation was also shown between PIC and MI ($r = 0.940$ and $p = 0.001 < 0.05$). A positive correlation of $p = 0.997$ and a significant $r = <0.0001<0.05$ are also shown in the relationship between EMR and MI. PIC, EMR, and MI are relevant to use as markers in genetic mapping and phylogenetic studies. It is in line with a study by Kolade et al. (2016), which reported that the RAPD method could determine genetic diversity and a population and detect plant mutation.

The amplification results of PCR-RAPD (Figure 6) show the thickness level and the number of bands that vary in each primer. Primer series of RAPD OPA-03 and OPB-17 has the highest number of bands compared to the other two primers and have varying thicknesses. The DNA quality of the different

Table 3
Results of polymorphism analysis and effectiveness of RAPD primers were noted

Primer	nB	nPB	% PB	PIC	EMR	MI
OPA 02	6	6	100	0.38	36	13.61
OPA 04	8	8	100	0.32	64	20.22
OPA 06	7	3	43	0.27	21	5.63
OPB17	8	7	88	0.34	56	18.87
Total	29	24	330	1.30	177	58.33
Average	7.25	6	83	0.32	44.25	14.58

Note. nB = Number of bands; nPB = Number of polymorphic bands, % PB = Percentage of the polymorphic bands; PIC = Polymorphic information content; EMR = Effective multiplex ratio; MI = Marker index

extractions in each sample determines the PCR result. The resulting band shows that a difference in thickness can also be affected by higher DNA concentrations, resulting in thick bands (Gusmiaty et al., 2016).

The NTsys analysis showed that the seven samples used in this study were divided into three clusters with a coefficient of 0.63. Cluster I consist of 24A (TRI 2024 Andongsili) and P15 (PGL 15 Pagilaran); Cluster II consists of 25A (TRI 2025 Andongsili) and G3A (Gambung 3 Andongsili); and Cluster III consists of G7A (Gambung 7 Andongsili), G9A (Gambung 3 Andongsili), and G7K (Gambung 7 Kayulandak). The coefficient of genetic similarities between individuals used ranges from 0.59-0.84. At the similarity level, 0.63 produces three clusters. This result suggests that the genetic distance of individuals in clusters is relatively far. Close similarity indicates low genetic distance, and far similarity indicates high genetic distance (Lucic et al., 2011). Far genetic distance indicates the presence of genetic diversity between the samples used in the study. The presence of high genetic diversity is one factor in the assembly of new superior varieties. Increased genetic diversity is done by utilizing germplasm that is available in nature or can be through crosses.

Each clone spreads into several clusters based on dendrogram data (Figure 7). For example, samples of Andongsili origin are distributed in several clusters. This result is probably due to variations in altitude, allowing recombination. Joshi et al. (2009) explain that genetic diversity can occur due to simple mutations and/or recombination during reproduction.

In Table 4, G9A (Gambung 9 Andongsili) and G7K (Gambung 7 Kayulandak) have a genetic similarity coefficient of 0.84, meaning that the two samples have a genetic similarity of 84%. While the samples P15 (PGL 15 Pagilaran) and G3A (Gambung 3 Andongsili), as well as P15 (PGL 15 Pagilaran) and G7A (Gambung 7 Andongsili), showed a coefficient of 0.45, which indicated that both had 45% similarities, respectively. Samples were taken from three locations, Andongsili, Kayulandak, and Pagilaran, with different altitudes. Andongsili is at an altitude of >1,000 masl, Kayulandak is at 1,000 masl, and Pagilaran <1,000 masl. The higher

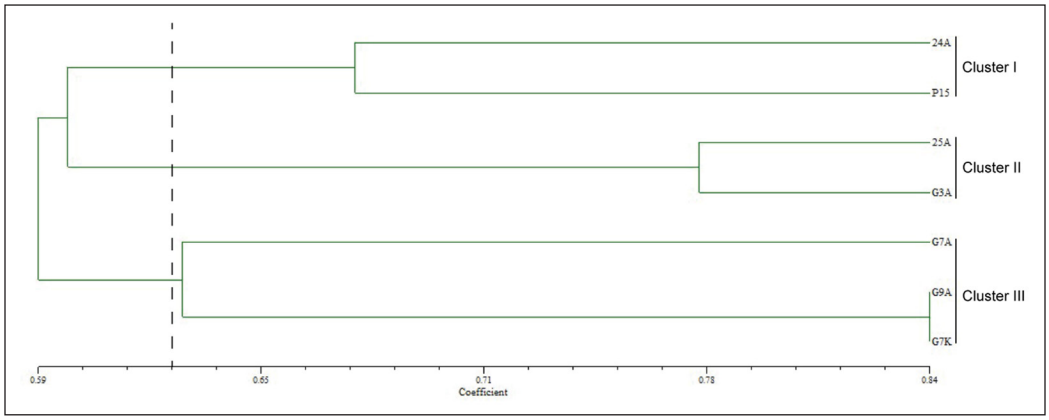


Figure 7. Dendrogram derived using data based on RAPD primers: 24A (TRI-2024 Andongsili), 25A (TRI-2025 Andongsili), G3A (Gambung 3 Andongsili), G7A (Gambung 7 Andongsili), G9A (Gambung 9 Andongsili), G7K (Gambung 7 Kayulandak), and P15 (PGL 15 Pagilaran), respectively

Table 4
Jaccard's coefficient similarity among Exobasidium vexans *samples*

	24A	25A	G3A	G7A	G9A	G7K	P15
24A	1						
25A	0.61	1					
G3A	0.65	0.77	1				
G7A	0.52	0.52	0.55	1			
G9A	0.65	0.65	0.55	0.67	1		
G7K	0.67	0.76	0.65	0.58	0.84	1	
P15	0.67	0.67	0.45	0.45	0.58	0.61	1

Note. 24A = TRI-2024 Andongsili; 25A = TRI-2025 Andongsili; G3A = Gambung 3 Andongsili; G7A = Gambung 7 Andongsili; G9A = Gambung 9 Andongsili; G7K = Gambung 7 Kayulandak; and P15 = PGL 15 Pagilaran

the coefficient value indicates that the two genotypes have a close or uniform correlation. Conversely, the lower the coefficient of genetic tendency has a genetic similarity (Martono & Syafaruddin, 2018). This result is in line with the dendrogram (Figure 7), in which the lowest coefficient value of 0.45 is PGL 15 Pagilaran and Gambung 3 Andongsili; PGL 15 and Gambung 7 Andongsili.

Other mechanisms such as horizontal gene transfer between *E. vexans* and tea plants can lead to evolution and environmental stress. The difference in the height of the place leads to differences in growth and the quality of the tea. The quality of tea is influenced by catechins, L-theanine, caffeine, flanovoles, theaflavins, and thearubigins that are generally obtained from a higher location.

Principal Coordinate Analysis (PCoA) (Figure 8) shows that sample codes P15 (clones PGL 15 Pagilaran) and 24A (TRI 2024 Andongsili clones) are in the same

cluster, sample codes G7A (Gambung 7 Andongsili clones) and G9A (Gambung 9 Andongsili clones) are in the same cluster, as well as sample code G7K (clone Gambung 7 Kayulandak), 25A (TRI 2025 Andongsili clone), and G3A (Gambung 3 Andongsili clone). Three large clusters were identified based on the seven genotypes of *E. vexans* analyzed by PCoA. The results of the PCoA analysis showed that genotypes in the same coordinates and adjacent positions were to be avoided as parents for breeding. Kumar et al. (2015) explain that the genotypes at the coordinates adjacent to its position are not to be crossed to obtain prospective segregation results in certain environments.

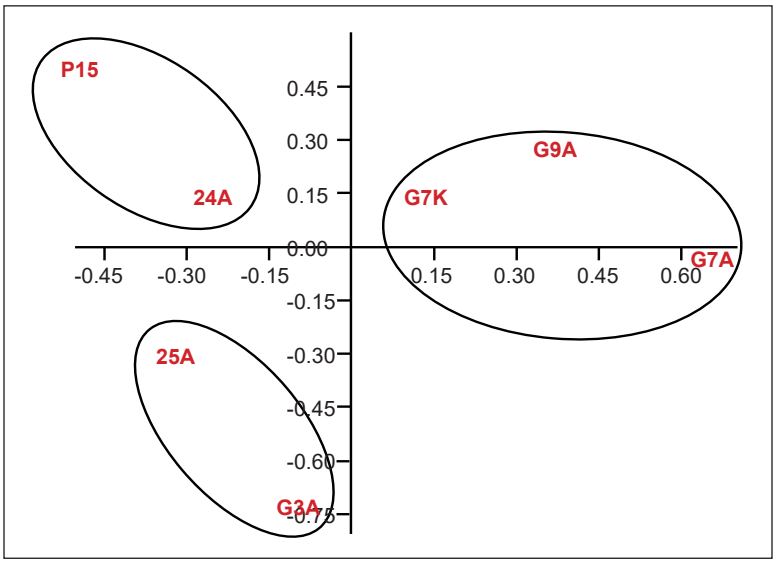


Figure 8. PCoA plot based on RAPD data: 24A (TRI-2024 Andongsili), 25A (TRI-2025 Andongsili), G3A (Gambung 3 Andongsili), G7A (Gambung 7 Andongsili), G9A (Gambung 9 Andongsili), G7K (Gambung 7 Kayulandak), and P15 (PGL 15 Pagilaran), respectively

CONCLUSION

Morphology of blister blight symptoms caused by *E. vexans* in between clones at each height showed no difference. The PCR-RAPD method using primary OPA-02, OPA-04, OPA-06, and OPB-17 can show the diversity of *E. vexans* samples between clones, and at coefficient 0.63 formed three clusters, cluster I consisting of TRI 2024 clones Andongsili and PGL 15 Pagilaran; cluster II clone TRI 2025 Andongsili and Gambung 3 Andongsili; and cluster III clone Gambung 7 Andongsili, Gambung 7 Kayulandak, and Gambung 9 Andongsili.

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Review Article

Passion Fruit—A Potential Crop for Exploration in Malaysia: A Review

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ABSTRACT

Passion fruit is a short-term crop with a life span of up to 2 years. Nowadays, passion fruit captures increasing demand in the global market. However, passion fruit is considered an underrated fruit in Malaysia as the production has not reached commercial cultivation. Highlighting the passion fruit as the next important commodity could rejuvenate the economy by disseminating equal benefits for both small and large-scale growers. This article provides a perspective on underlining the fruit to be explored as a commercial commodity. The fruit consists of three main components: juice, seed, and peel. Each of the fruit components has unique properties that can benefit multiple industries. In addition, strategies for successful passion fruit planting are also emphasised by farm management until the processing line produces high-quality fruit that can penetrate the global market. Therefore, a comprehensive review of passion as an essential crop could benefit Malaysia's agriculture and processing industries.

Keywords: Agricultural industry, economy, fruit quality, market expansion, natural product, passion fruit

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INTRODUCTION

Passiflora edulis is a perennial vine plant belonging to the Passifloraceae family. There are 18 genera under the Passifloraceae family that consist of 530 species, where 50–60 species are edible (Ghada et al., 2020). There are two types of passion fruit involved in global cultivation which are purple passion fruit (*Passiflora edulis*

Sims) and yellow passion fruit (*Passiflora edulis* f. *flavicarpa* Degener) (Md Nor et al., 2021a). The common name of passion fruit varies according to country. In Spanish, gulupa refers to purple passion fruit, while granadilla, parcha, and maracuyá refer to yellow passion fruit. Purple passion fruit is known in Portugal as maracuja, peroba, and maracujazeiro, whilst in France, it is known as grenadille and couzou (Pineapple Research Station, 2010). There is no specific name to distinguish between the purple and yellow passion fruits in Malaysia since both are called markisa. Morphologically, both fruits are round with leathery skin and contain aromatic, juicy pulp with small black seeds (Md Nor et al., 2022). Purple and yellow passion fruits can be differentiated by the plant's growth performance and fruit properties. The purple passion fruit plant is more resistant to cold injuries, while the fruit juice is less acidic and superior in aroma and flavour, making it highly palatable as dessert fruit (Azizah, 2011). *Vice versa*, the yellow passion fruit plant is not chilling resistant to a more vigorous vine, and the fruit has more acidic juice making it utilised highly in food processing industries (Md Nor et al., 2021b). Globally, purple passion fruit is mainly cultivated in East Africa, Venezuela, Brazil, Peru, Ecuador, and Colombia, while yellow passion fruit is primarily grown in South America (Kishore et al., 2006). The growing passion fruit in Malaysia is still underrated because it is used mainly for regional cultivation and has not yet reached commercial levels.

Market Potential of Passion Fruit

The passion fruit market can be segmented into North America, Europe, Asia Pacific, South America, the Middle East, and Africa (Altendorf, 2018). In 2019, Europe became the largest market share of passion fruit, followed by South America and North America. In Europe, passion fruit is highly traded as fresh, exotic fruit (Arias et al., 2020). The import value experienced a 40% increment over the past five years, with 42 million Euros in 2019, and this has made European countries, which Germany dominates, become the biggest importers (Center for the Promotion of Imports [CBI], 2021). This value is projected to continually increase in the upcoming years since there is high demand for fruit such as desert fruit and fresh juice. Since the beginning of 2020, when the Corona Virus Disease-2019 (COVID-19) pandemic hit the global, consumers physiologists found out consumers' behaviour changed with an attempt to eat a healthy diet with plenty of fruit and vegetables (Borsellino et al., 2020). The consumers stockpile food and confer for longer shelf-life nutritious food. In the European market, one of the fruit products that grabbed high demand during the panic buying was passion fruit puree (The Insight Partners, 2020). Passion fruit puree retains its full flavour and colour of fresh fruit. It is manufactured from fully ripened fruit that has been frozen, providing cost-effective products with a longer shelf life alternative to fresh fruit (Vaillant et al., 2001). Between 2004 and 2007, fresh passion fruit's price was around US$250/metric tonne in US

and European markets (Altendorf, 2018). The price has increased with fresh fruit and puree rated at US$400–600/metric tonne (Anonymous, 2022). The global market analysts have predicted that due to the pandemic, the demand for passion fruit puree will continue to rise until 2027 (The Insight Partners, 2020). The expanding global market price of passion fruit is remunerative and estimated to bring extra revenues to the producers. Most global passion fruit producers belong to tropical and subtropical countries (Table 1). Since Malaysia is tropical, the climate could be conducive to passion fruit cultivation.

Table 1

The different geographical features of global passion fruit producer

Country	Production (ha)/year	Type of cultivation	Climate	Light/ solar radiation (hour/ day)	Altitude (m)	Temperature (°C)	Rainfall (mm)	References
Ecuador	948,100	Plantation farming	Subtropical	12	51–479	23–25	64-2,658	Viera et al. (2020)
Brazil	150,000	Plantation farming	Subtropical	10–13	13–1,000	17–26	1,300-1,844	de Jesus et al. (2018)
Indonesia	114,600	Plantation farming	Tropical	12	195–2,000	28–32	2,600-3,000	Nadja et al. (2019)
India	78,000	Plantation farming	Subtropical	12	800–1,500	18–23	1,000-2,500	Tripathi et al. (2014)
Colombia	60,000	Plantation farming	Subtropical	5-13	0–2,200	15–28	800-1,800	Fischer et al. (2018)

Passion fruit plants grow well in regions with the temperature between 28 °C–33 °C and 60%–70% relative humidity. This environment will enable the flower's stigmas to remain hydrated and adhesive for effective pollination, thus producing fruit with a sweet juice taste and content (Fischer & Miranda, 2021). The flower is photoreceptive and only blooms after adequate sunlight. The agro-climatic conditions of Malaysia are tropical, with an average daily temperature of 27 °C–32 °C and 60%–70% relative humidity, while its annual precipitation is 2,000 mm to 2,500 mm (Malaysian Meteorological Department [MetMalaysia], 2022). In addition, Malaysia receives equal days and nights throughout the year, allowing the plant to flower and fruit optimally (Tripathi et al., 2014). The optimal ecosystem should be grasped so that Malaysia can produce the fruit on a mass scale.

Malaysia has around 310,000 acres of fruit-planting land. The output stands at 1.8 million metric tonnes (mt) with a USD127.8 million export value to Singapore, Hong Kong, and the Middle East. Malaysia continues to be a net importer of fresh and processed fruits, with fruit imports valued at around USD174 million (Salleh

& Yusof, 2006). In the late 60 and early 70s, Malaysia first positioned pineapple as a commercial commodity for export (Jaji et al., 2018). Till now, the variety of commercial fruits has grown to more than 10 fruits. In 2017 commercial fruits include banana, pineapple, durian, watermelon, and guava, with a production of around 84,288–350,493 ha/year (Figure 1).

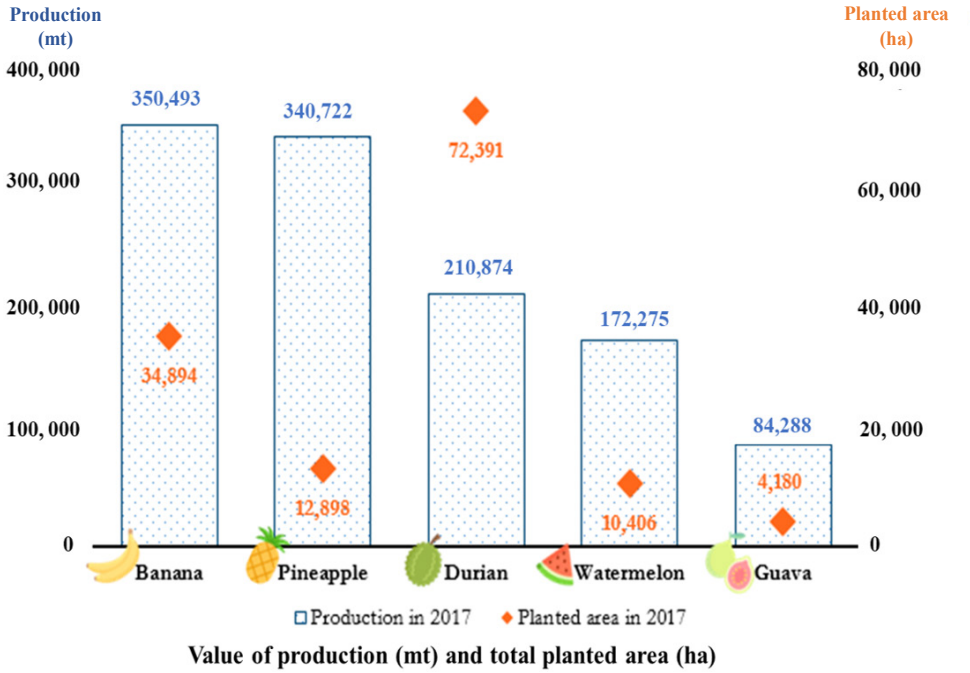

Figure 1. Different types of commercial fruits in Malaysia that were produced in 2017 [Source from Tumin and Shaharudin (2019)]

Among fruit planting areas, durian has the largest acreage with 72,391 ha. In 2019, Malaysia was only focusing on durian (*Durio zibethinus*), jackfruit (*Artocarpus heterophyllus*), papaya (*Carica papaya*), watermelon (*Citrullus lanatus*), and banana (*Musa* spp.) as export commodities (Rozhan, 2019). Nevertheless, due to inadequate information about its feasibility, passion fruit planting remains an underutilised crop in Malaysia. The fruit is not even listed as an essential commodity in Malaysia (Ministry of Agriculture and Food Industries [MAFI], 2022). Even though the passion fruit is not particularly popular in Malaysia, it has a competitive market price compared to other commercial commodities (Figure 2).

Among these fruits, durian poses the most premium market price with US$3,500–12,500/metric tonne, while papaya values US$317–333/metric tonne, watermelon at US$300-321/metric tonne, and jackfruit at US$200–300/metric tonne. An exception to durian, passion fruit has a higher export value than other fruits, priced at US$400-600/metric tonne. Although durian fruit

Commodity	Gross production value (current thousand USD)	Minimum market price (USD per tonne)	Maximum market price (USD per tonne)
Passion fruit	-	400	600
Papaya	20,575	317	333
Watermelon	43,108	300	321
Jackfruit	-	200	300
Durian	-	350	1,250

Figure 2. The commercial market price of tropical fruits in the global market in 2017–2020 [Source from Food and Agriculture Organization Corporate Statistical Database (FAOSTAT) (2022)]

could give a good return, it is a long-term crop in Malaysia. The tree takes four to eight years to bear fruit (Ketsa et al., 2020). In addition, the durian tree is tall and big, which takes up a large area of space, making crop management difficult (Radchanui & Keawvongsri, 2017). The long-term investment of time and effort makes the durian's price premium. Nevertheless, the investment cannot assure economic guarantee to the growers as there are substantial risks that can cause losses, especially when fungal diseases and floods infect the trees. Due to the intimidating likelihood, durian growers require big capital for intricate plant management and tolerate risky investments.

Venturing to passion fruit as the next enlightened crop could offer profitable investment since it is getting high demand globally. The plant is a vigorous climbing vine crop that can grow quickly. Previous research shows that the plant starts to bear fruit within six months after transplanting, and the crop's life span is up to 2 years (Md Nor & Ding, 2019). Passion fruit is a short-term cash crop that could bring a rapid return to growers, thus offering a less risky investment. Venturing into passion fruit cultivation is less capital intensive but captures a higher market price that could bring a better return (Nsubuga, 2021). Reflecting Brazil, the world's largest commercial passion fruit producer, the production is 7,613 kg/ha, which leads to a net profit of US$3,519.22, a benefit-cost ratio of 2.5, and a profitable index of 177.5% (da Silva et al., 2020). The profitable index of passion fruit is higher than bananas, valued at 141.58% (Magbalot-Fernandez &

Montifalcon, 2019). Cultivating the passion fruit in mass-scale production could bring economic growth to Malaysia as the fruit offers remunerative profit.

About 97% of smallholders in Malaysia lack capital investment and limited land size (Arshad, 2016). Large-scale growers are usually established by large-scale organisations that are professionally managed and have a substantial amount of capital capable of in investing both short and long-term crops (Ng, 2016). Nonetheless, the small-scale farmers grab a small chance to invest a substantial amount of money in a long-term commodity like durian. Offering the passion fruit in commercial planting could provide a better opportunity for small-scale growers to improve their socio-economy (da Silva et al., 2020). This strategy may help the Malaysian agricultural industry to build resilience in rural communities and improve the socio-economy. The small-scale farmers have significantly impacted the Malaysian economy, contributing 23% of total export earnings and 7.2% of gross domestic product (GDP) (Ng, 2016). In 2020, Malaysia's economy had contracted by 5.6% due to the pandemic crisis. Therefore, for the Twelfth Malaysia Plan (12MP) (2022) from 2021–2025, the government has outlined an agenda for holistically restoring economic stability. The agriculture sector is being highlighted for the next five years to rejuvenate the Malaysian economy, which includes the production of vegetables and fruits. Positioning the passion fruit for commercial cultivation in Malaysia could positively impact the agricultural sector, restoring its economic stability.

Passion Fruit as Multifunction Fruit for Industrial Usage

Botanically, passion fruit belongs to pepo, a type of berry. Upon pollination, the flower ovule is developed into a seed while the walls of the ovary are transformed to peel (Md Nor et al., 2021b). Morphologically, the tissues can be segregated into pericarp (peel) and endocarp (seed and juice) (Figure 3).

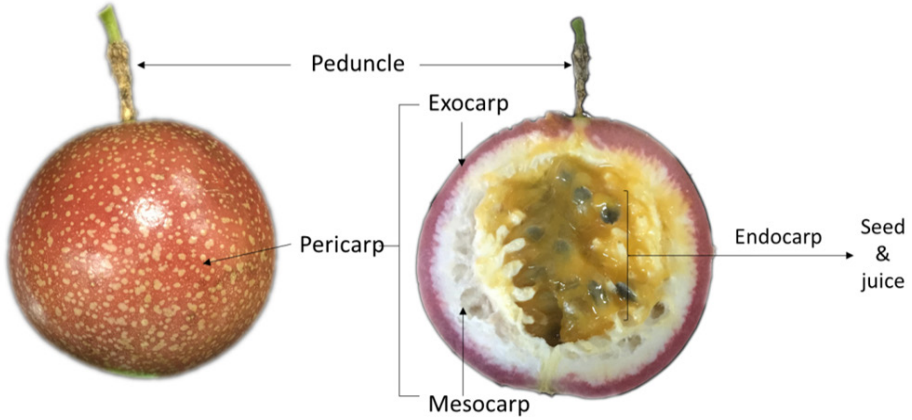

Figure 3. Morphological organisation of a passion fruit tissue. Pericarp refers to the peel composed of exocarp (outer layer with purple colour) and mesocarp (white spongy layer). Endocarp refers to the flesh that is composed of seed and juice

In the fresh fruit industry, only the seed and juice are edible, while the peel is normally discarded. Nonetheless, due to the unique benefits of each tissue, the whole fruit compartment can be fully utilised for diverse industrial products (Table 2). Passion fruit juice has a distinct sweet-sour and fruity flavour with notable amounts of potassium, copper, phosphorus, iron, and vitamins A, B, and C (He et al., 2020). Passion fruit juice extracts show a wide range of potential health effects and biological activities: antioxidant, anti-hypertensive, antidiabetic, anti-tumour, and hypolipidemic (Guimarães et al., 2020). Besides, it is considered a low-calorie fruit, making its consumption suitable for those on a diet (He et al., 2020). Daily consumption of fresh passion fruit juice was also reported to be non-toxic. This opportunity makes the passion fruits economically important worldwide as they have a great demand to be marketed for fresh consumption, food, and beverages.

Table 2

Summary of passion fruit's functional properties and its potential industrial applications

Compartment	Functional properties	Industrial applications	References
Juice	Nutritious and lucrative taste	Fresh consumption, food, beverages, and confectionaries products	Zhu et al. (2017)
Seed	Rich in essential fatty acid	Premium edible oil	He et al. (2020)
	Excellent source of fibre	Natural food fibre ingredient	Liu et al. (2008)
	Rich in phenolic and flavonoid	Food supplements	He et al. (2020)
	Source of stilbenes	Medicine for cancer and diabetic	Yamamoto et al. (2019)
	Tyrosinase inhibitory effects	Cosmetic	Yepes et al. (2021)
Peel	Rich in fibre content	Thickener and gelling agent	Lima et al. (2016)
	Excellent rheological property	Flour with medicinal function	Lima et al. (2016)
	Good antioxidant property with antimicrobial activity	Natural food preservatives	Ramli et al. (2020)

Passion fruit juice is wholly used in the food and beverages industry. The passion fruit juice is normally extracted from the pulpy seed and processed as clarified juice or juice concentrates. Production of clarified juice involves extraction from the pulpy seed. Then, the clarification process is conducted by treating the juice with pectinase and afterward being filtered, pH adjusted, sweetened, packed, and sterilised (Zhu et al., 2017). A similar processing step is conducted for juice concentrates but with additional steps in which 50% of water content will be removed (Vaillant et al., 2001). Concentrates are not intended for direct consumption as they can be reconstituted and incorporated in the desert, carbonated drinks, ice pop, ice cream, dessert, and multiple confectionery products. Passion fruit juice concentrates are now even available in powdered form, allowing for greater applications and extended shelf-life (Borrmann et al., 2013). The functionality of passion fruit juice in the food industry is still expanding as food technologists actively develop innovative products. Recent research shows that the juice can be fabricated into passion fruit juice-enriched set yogurt (Ning et al., 2021), nutritious jelly drinks (Agnes et al., 2021), and probiotic drinks (Guedes et al., 2021).

The passion fruit seed is equipped with high sodium contents, followed by magnesium, phosphorus, potassium, and calcium (He et al., 2020; Lourith & Kanlayavattanakul, 2013). Around 20% of oil can be extracted from the seed, mainly comprised of 70% linoleic acid, followed by oleic acid, palmitic acid, and a trace amount of linolenic acid (Liu et al., 2008). This compound makes the seed a great source of essential fatty acids that can function in the food industry. Furthermore, premium edible oil can be extracted from passion fruit seed as it is predominantly constituted of unsaturated fatty acid, where linoleic acid exceeds 60% (Liu et al., 2008). Apart from that, the seed is also an excellent source of fibre. A study by Chau and Huang (2004) has proven that passion fruit is rich in insoluble dietary fibre (99.96%) composed of pectin, cellulose, and hemicellulose with the capability of absorbing glucose and retarding the amylase activity. The findings also suggest that the seed fibre can enrich the formulation of healthy dietary snacks.

The passion fruit seed is also rich in antioxidants with high phenolic and flavonoid contents that work as active compounds where it can be formulated as food supplements for healing life-threatening diseases such as cancer, immunodeficiency disorders, and cardiac disorders (He et al., 2020). Passion fruit's seed extract is rich in stilbene, such as piceatannol and scripusin, which have various physiological effects where these compounds can provide a novel anticancer strategy for preventing and treating diseases (Yamamoto et al., 2019). The piceatannol from the seed improved insulin sensitivity in obese patients and potentially reduced type 2 diabetes (Kitada et al., 2017). The oral intake supplement made by passion fruit seed extract, which is rich in piceatannol, improves dry skin, and reduces fatigue among women, who suffer

from these problems (Maruki-Uchida et al., 2018). In addition, the tyrosinase inhibitory effects of the seed extract can be beneficial for developing skin-lightening cream, sunscreen, and anti-ageing cream (Yepes et al., 2021).

Like the passion fruit's seed, the peel composed of high fibre makes up 60%–80% of the fruit's total weight (He et al., 2020). Pectin is well known as a functional food ingredient that grabbed high commercial value by its technological properties. The pectin can be used as a thickener, gelling agent, and stabiliser in formulating foams and emulsions (Elizabeth et al., 2019). Due to this technical quality, pectin extracted from the passion fruit can be used to prepare jellies, jams, fruit juice, and formulation of concentrated dairy products such as yogurt (Elizabeth et al., 2019). Furthermore, passion fruit pectin is safer for human health because it is a natural element that can improve digestive system function and boost satiety (Freitas et al., 2016). Ingesting the passion fruit pectin leads to gel formation in the gastrointestinal tract. Due to high flavonoid contents, the gel later inhibits the gastric absorption of sugar and amino acids in the small intestine, reducing postprandial blood glucose in controlling diabetes response (Espinal-Ruiz et al., 2016).

Passion fruit's peel can be processed directly as flour. Technically, the passion fruit's peel flour can replace the commercial hydrocolloids. The flour can be processed with a low-cost procedure but obtain similar technological characteristics to commercial hydrocolloids in stabilising, emulsifying, thickening, and gelling power (Lima et al., 2016). Therefore, passion fruit peel flour can be considered a high nutritional ingredient. Study shows that the intake of passion fruit peel flour can effectively lower cholesterol and triacylglycerides levels in HIV patients (Marques et al., 2016). Furthermore, *in vivo* study shows that direct intake of passion fruit peel flour can improve short-chain fatty acids production, ameliorate antioxidant status, modulate the microbiota, and improve insulin sensitivity (Lima et al., 2016). In addition, the extract obtained from passion fruit peel has great potential application in the meat industry as it can be developed as a natural additive to control oxidation and spoilage microorganisms due to its antioxidant and antibacterial properties (Ramli et al., 2020).

Strategies for Successful Passion Fruit Planting

It is critical to assist growers in producing high-quality fruit that can penetrate the global market. Strategies are needed to promote this fruit among Malaysian growers so that growers are willing to invest in planting passion fruit. The first strategy is determining the optimal stages to cater to different distances of markets (Md Nor & Ding, 2020). Estimating the fruit harvesting stage is vital for ensuring the fruit quality is optimal when it reaches consumers. The harvesting stage significantly impacts fruit eating quality and the post-harvest life of manufactured products (Camargo et al., 2017; Mohammad & Ding, 2019; Tee et al., 2012). For a fruit grown in tropical

regions with a uniform climate throughout the year, calendar dates that depend on days from flowering to fruit set can be used as a reliable guide to predicting its optimal harvesting stage. The flowering duration can predict the relative growth period of fruit to maturity (Camargo et al., 2017). This method is applicable since the time of flowering, and fruiting greatly depends on temperature, humidity, precipitation, and radiation. The uniform climate makes the harvesting stage relies on calendar dates to become successful, as applied in a few countries such as Malaysia, Vietnam, and Thailand to predict fruits' maturity (Uda et al., 2020).

Ensuring fruit is harvested at its optimal harvesting stage, it should achieve its minimal level of acceptability during harvesting. The grading requirement that outlines the minimal acceptability of fruit for trading is known as maturity indices. The maturity indices are maturity declaration to avoid selling immature or overmature fruit, undermining consumer confidence (Yadav et al., 2014). The common maturity indices for fruit are summarised in Table 3. The physical characteristics (size, colour, texture), chemical (soluble solids concentration, organic acid, and sugar content), and physiology (ethylene and respiration) were found to have good correlations with the stage of fruit development (Mohammad & Ding, 2019).

Table 3

Criteria in ascertaining the fruit maturity indices (Paltrinieri, 2014)

Criteria	Fruits
Physical properties	
Development of abscission layer	Some melons, apples, and feijoa
Surface morphology and structure	Grapes and tomatoes (cuticle formation) Some melons (netting formation) Some fruits (glossiness)
Size	All fruits
Specific gravity	Cherries, watermelons, and potatoes
Shape	Banana (fingers angularity) Mangoes (full cheeks)
Textural properties - Firmness - Tenderness	 Apples, pears, stone fruits Peas
External colour	All fruits
Internal colour and structure	Tomato (surface with a jelly-like colour) Some fruits (flesh colour)
Cellular structure	
Angularity of cell and cell size at mesocarp layer	Banana and karanda fruit

Table 3 *(Continue)*

Biochemical properties	
Starch content	Apples and pears
Sugar content	Apples, pears, stone fruits, and grapes
Acid content, sugar/acid ratio	Pomegranates, citrus, papaya, Melons and kiwifruit
Juice content	Citrus fruits and avocados
Astringency (tannin content)	Persimmons dates
Physiological properties	
Internal ethylene concentration	Apples and pears
Mean heat units during development	Peas, apples, and sweet corn

The indices cover fruits' physical, cellular, biochemical, and physiological properties. Ideally, fruit maturity indices involve non-destructive measurement, simple to measure by producers, handlers, and quality control personnel, which are readily performed in the field with non-sophisticated instruments (Lina et al., 2014). Specific criteria have been outlined for passion fruit maturity indices released by commercial producers such as America, Brazil, and Kenya (Ghosh et al., 2017). The indices are simplified in Table 4. These indices can be inferred as guidelines for establishing the optimal harvesting stage of passion fruit in Malaysia. The primary step for establishing maturity indices is to study the physicochemical properties of fruit during growth and development (Camargo et al., 2017; Mohammad & Ding, 2019; Tee et al., 2012). Nevertheless, this study is still lacking in Malaysia; therefore, it is crucial to ratify it first.

Table 4

Maturity indices of passion fruit in global trading (Codex Alimentarius, 2014)

Indices	Descriptions
Size (Diameter)	Small (5 cm) Medium (6.5 cm) Large (8 cm)
Peel colour	Green—not ripen 50% purple colour—acceptable for market 75% purple colour—acceptable for market 100% purple colour—highly desirable
Soluble solids concentration	13–18 °Brix

The second strategy for commercial planting passion fruit is to control the pests and diseases of the plant. Fungal infestation is a major threat to the commercial cultivation of passion fruit. Fungal infestations such as *Fusarium oxysporum*, *Phytophthora cinnanomi*, *Phytophthora nicotinae*, and *Colletotrichum gloeosporioides* can cause various diseases such as fusarium wilt, collar rot, anthracnose, and crown rot on passion fruit (Melo et al., 2019). Pale-green leaves, mild dieback, chlorosis, leaf drop, plant wilting, and death are common signs of diseases too. In 1960, the passion fruit plant was once cultivated on a commercial scale in Malaysia. However, the project was unsuccessful due to the fungal disease outbreak (Chai, 1979). Nevertheless, research in agriculture has undergone tremendous advancement in this era. The use of chemical and bio-fungicide was proven effective in controlling bacterial and fungal diseases in plants (C. Wang et al., 2021; Ons et al., 2020). Therefore, the historic failure of commercial passion fruit planting in Malaysia cannot be interpreted as a hurdle for the current implementation. Therefore, a pilot-scale study on passion fruit cultivation must be executed to evaluate the project's feasibility before the implementation of full-scale production.

The third strategy for commercial planting passion fruit is efficient post-harvest management. An effective management system can ensure the harvested product meets the market/consumer expectations in terms of volume, quality, and other transaction features, such as nutrition and product safety (Md Nor & Ding, 2020). Passion fruit is a climacteric fruit. The beauty of climacteric fruit is that the fruit can be harvested at early maturity and ripened during transportation and distribution (Wang & Sugar, 2015). The application of 1-methylcyclopropene (1-MCP) is a standard method to prolong the post-harvest life of passion fruit in a commercial by inhibiting the production of endogenous ethylene in the fruit (Yumbya et al., 2014). Nevertheless, the tricky part of applying 1-MCP is that it must be applied when the fruit's endogenous ethylene is neither too low nor too high to allow appropriate fruit ripening. Therefore, before applying this technique, the physiological feature of passion fruit needs to be understood to allow effective treatment that can produce optimal fruit quality.

Besides, optimal keeping temperature for passion fruit needs to be adapted too. The passion fruit is known to be chilling and sensitive. Storing the Malaysian grown passion fruit at 5 °C causes the fruit to develop chilling injuries (Md. Nor et al., 2021a). On the other hand, the yellow passion fruit was found to tolerate well with 10 °C as the fruit achieves extension in post-harvest life for 21 days (Md Nor et al., 2021a). Modified atmosphere (MA) packaging can also become an effective way to lengthen the fruit's post-harvest life. Packing passion fruit in the MA environment will allow the fruit to gain at least 14 days of shelf-life extension (Yumbya et al., 2014). Other than that, the application of biopolymer coating may also be effective

in extending the post-harvest life of passion fruit. Currently, limited research is available for passion fruit coating (Md Nor & Ding, 2020). Therefore, venturing into coating formulation specifically for passion fruit can become a good research niche in preparing the Malaysian passion fruit for commercial cultivation.

Passion fruit cultivation can also be oriented to expand beyond the fresh fruit as well as the food and beverages industry. It is because fruit can be the principal source of medicine for new drug development in addition to agrochemicals, cosmetics, and food ingredients (He et al., 2020). Nevertheless, this process needs integration between the farm production and natural product industries. The natural product industry is an important sector in the economic growth of a country. The sector covers the production of cosmetics, herbal medicine, pharmaceutical products, and natural food ingredients (Atanasov et al., 2021). According to the study made by BCC research company, the global plant-derived natural products were valued at US$ 22.1 billion in 2012, and sales are expected to expand to US$ 26.6 billion by 2017 at a compound ground rate (CAGR) of 3.8% (Krause & Tobin, 2013). For instance, the European country (EU) has become the world's leading producer of sugar beets, with a cultivation area of 1.74 million hectares targeting the fruit for fresh consumption, sugar production, and natural dye production (Haß, 2022; Popescu et al., 2021). In America, Solanaceae *Datura stramonium* are planted commercially to produce tropane alkaloids, which function as starting material in the production of different pharmaceuticals products (Moreno-Pedraza et al., 2019). *Morinda citrifolia* are planted commercially in India as a dietary supplement instead of being processed as juices, powder, flavouring, and colouring agents for food and drinks (Thorat et al., 2017).

As discussed in previous sections, passion fruit has multifunction usage in pharmaceutical, functional food ingredients, and cosmetic industries. Extended passion fruit products, such as passion fruit peel flour, pectin, seed essential oil, and flavonoid passion fruit extract, have been commercialised primarily in America and China (Anonymous, 2022; He et al., 2020). Production of extended passion fruit products could offer remunerative income for the country since the product segmentation fit various technological industries willing to pay for exclusive prices (Lang & Rodrigues, 2022). The natural product industry will benefit good return on investment as raw materials will become cheaper if Malaysia starts to implement commercial cultivation. Symbiotic interaction between the farm production and natural product industry is essential to be inaugurated. Consequently, the Malaysian economy will gain dual revenue from both industries.

CONCLUSION

Commercial passion fruit cultivation in Malaysia is a propitious plan of action. The rising global demand for fruit should be viewed as an opportunity to boost

the economy. The commodity can be endeavoured by small- and large-scale growers. The fruit can be classified as multifunction, which can be exploited beyond the orthodox food and beverages industry. With the advancement of technology, juice, seed, and peel can be converted to various extended passion fruit products. A commercial planting strategy includes effective management, which begins with producing high-quality fruit. After harvest, proper post-harvest handling should be applied to ensure the fruit maintains its quality until it reaches the consumer. Integration between farm production and the natural product industry needs to be established to penetrate passion fruit as extended passion fruit products.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Review Article

Rumen Fluke in Cattle and Buffaloes in Asia: A Review

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ABSTRACT

Rumen fluke is a parasitosis that infects ruminant animals across a wide geographical range of countries. It is a severe infection in temperate and tropical climate regions of Asia, Australia, Africa, and Europe, which cause significant economic losses. In this review, the available information to date on rumen fluke species infecting cattle and buffaloes in Asian countries is evaluated. The citation search was performed through specific keywords, literature published from 1964 to 2021, retrieved from electronic databases: Scopus, Web of Science, Pub Med, Education Resources Information Center (ERIC), Science Direct, Elsevier, and Google Scholar. Twenty-six (26) rumen fluke species belonging to two families: Paramphistomidae 61.5% (16/26) and Gastrothylacidae 38.4% (10/26), were reported in cattle and buffaloes in fourteen Asian countries. *Paramphistomum cervi* and *Cotylophoron cotylophorum* are the most prevalent species with broader distribution in countries than the other genera. The coprological prevalence varies from 0.8% to 98.17% and 0.86% to 78.4% in cattle and

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buffaloes, respectively. The prevalence of rumen fluke by fluke counts method range between 6.45% to 90.6% and 4.29% to 75.07% in cattle and buffaloes, respectively. The sedimentation method and fluke count are reliable tests for detecting rumen fluke in live and slaughtered animals. In conclusion, the rumen fluke should be considered a critical production disease that affects cattle and buffaloes in Asia. Further studies are necessary to determine the rumen fluke-snail associations, develop diagnostic tests to detect prepatent infections in the definitive host, determine the economic importance of rumen fluke, and determine the efficacy of different anthelmintic in the treatment of patent infections in the definitive host.

Keywords: Asian countries, cattle and buffalo, epidemiology, prevalence, rumen fluke

INTRODUCTION

Helminth infections are ubiquitous and seasonal, reside in the digestive tract of ruminants, and are responsible for significant impacts on animal production (Charlier et al., 2014). Bovine is most susceptible to chronic diseases because most bovine production systems are pasture-based (Sargison et al., 2016). Rumen fluke infection is present worldwide in the temperate and tropical climate regions of Asia, Africa, Europe, and Australia (Harizt et al., 2021; Huson et al., 2017; Pfukenyi & Mukaratirwa, 2018) and infects domestic ruminants, such as cattle, buffaloes, sheep, goat and some wild ruminants, which are caused by digenean fluke (Income et al., 2021; Pfukenyi & Mukaratirwa, 2018). Nowadays, rumen fluke is an emerging parasitic infection in European ruminant animals (Huson et al., 2017). Rumen flukes are responsible for poor feed conversion efficiency leading to weight loss or decreased milk production, which causes significant economic losses (Huson et al., 2017). In tropical and subtropical countries, the prevalence of rumen fluke is high because the climatic condition is suitable for intermediate host mud or freshwater snail to grow and complete the parasite life cycle (Gordon et al., 2013; Hajipour et al., 2021). In Asia, rumen fluke infections due to *Paramphistomum* spp. widespread with high prevalence in South and Southeast Asia (Debbra et al., 2018).

In 1970, the first published information on rumen fluke emerged, reporting the adult fluke in the rumen of European red deer (Sey, 1982). The terminology was changed several times before Fischoeder created the genus *Paramphistomum* in 1903. They belong to several families such as Paramphistomoidae superfamilies, Paramphistomidae, Gastrothylacidae, Gastrodiscidae, Oliveriidae, Balanorchiidae, and Stephanopharyngidae (Taylor et al., 2007). Different paramphistomoid species, particularly those of the Paramphistomidae and Gastrothylacidae families, cause amphistomosis in ruminants (Lotfy et al., 2010). Immature fluke causes severe infection in the small intestine of hosts (Horak, 1971).

Despite being historically viewed as minor importance in ruminants in Asian

countries, recent studies suggest that rumen fluke has increased in Asian countries and is more significant than fascioliasis in the United Kingdom (Huson et al., 2017). However, there is insufficient information about the biology and epidemiology of rumen fluke in Asia. Knowing the basic biology and epidemiology and how it interacts with the ruminant's host is essential. Since there has not been a review on rumen flukes in cattle and buffaloes in Asia, the information that is currently available on the species of rumen flukes that infect large ruminants (cattle and buffaloes) in Asian nations, intermediate snail hosts, and rumen fluke epidemiology has been analysed.

Description

Rumen flukes are small, conical (pear-shaped) maggot-like flukes, and the size is about 3 to 20 mm in length and 1.5 to 7 mm in width in domestic ruminants (Taylor et al., 2007). The larval stages are much smaller, at less than a millimetre in length, and fresh specimens have a pink colour (Toolan et al., 2015) (Figure 1). There are two suckers known as an anterior sucker and a posterior sucker (acetabulum). The digestive system of rumen fluke comprises the mouth, pharynx, oesophagus, and two intestinal caeca (Tandon et al., 2014) (Figure 2).

Figure 1. Adult rumen fluke attached to rumen papillae

Life Cycle

Rumen flukes need ruminants as definitive hosts and snails as intermediate hosts to complete their life cycle (Taylor et al., 2007). The adult flukes shed eggs in the stomach of infected animals, and eggs are passed to the environment with the faeces. Rumen fluke eggs are large and measure around 160 × 90 µm with a thin or thick eggshell that operculated with a distinct operculum (Figure 3). The eggs hatch under suitable conditions (temperature and humidity), and miracidia are released from

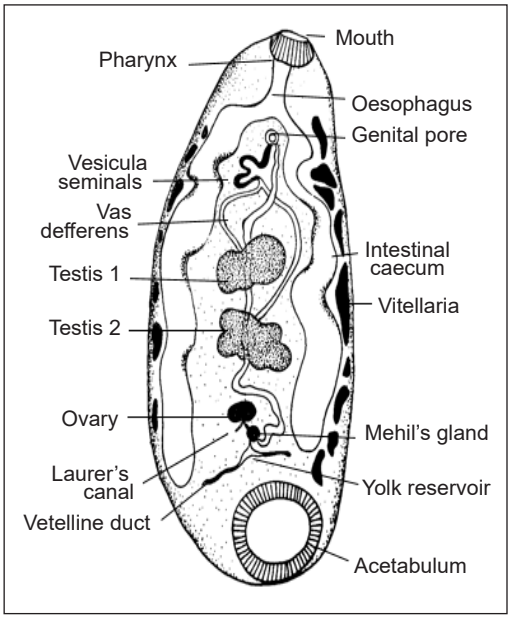

Figure 2. General diagram of a rumen fluke

the eggs (Bhatia et al., 2016; Hajipour et al., 2021). Miracidia searched for a suitable freshwater snail within 24 hours (de Waal, 2010). The miracidium reaches the soft tissues of the molluscan host (snail), either by innate behaviour, random chance, or chemotaxis (Tandon et al., 2014). Three stages (sporocyst, redia, and cercaria) develop inside the snails from 26 °C to 30 °C. Cercaria is released from the snails and encysts to metacercaria on the plant, which remains viable for up to six months (de Waal, 2010; Horak, 1971; Huson et al., 2017). Finally, the definitive host ingests metacercaria on the plant, excystment occurs in the small intestine, and juvenile fluke hatch and attach to the mucosa, which later (3 to 6 months) migrate to the rumen (de Waal, 2010). In the rumen, the fluke attaches to the ruminal pillar's surface, develops to the adult stage, and sheds the eggs (Taylor et al., 2007) (Figure 4).

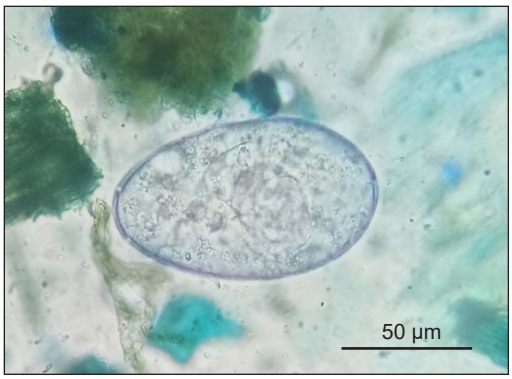

Figure 3. Rumen fluke egg was photographed at 400× by light microscope

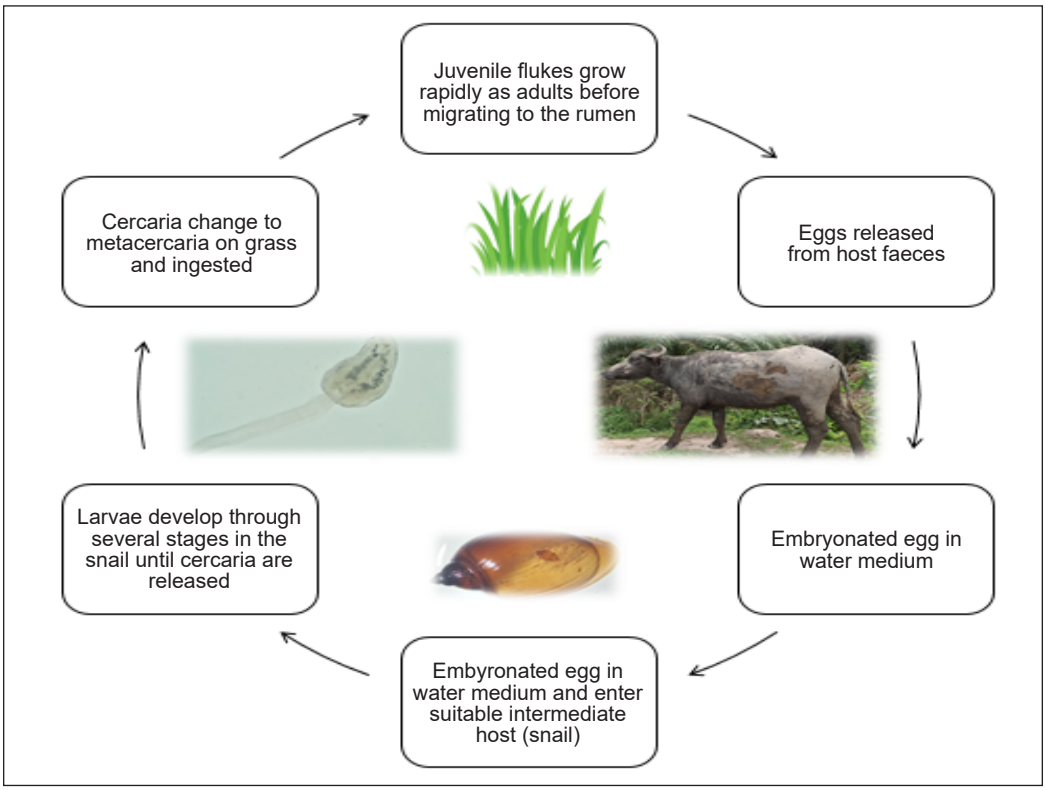

Figure 4. The life cycle of rumen fluke

Rumen Fluke Species Infecting Cattle and Buffaloes in Asian Countries

Rumen fluke species in cattle and buffaloes in Asian countries belong to two families: Paramphistomidae and Gastrothylacidae, and 26 species in cattle and buffaloes are as follows: *Paramphistomum cervi*, *Paramphistomum gotoi*, *Paramphistomum epiclitum*, *Paramphistomum gracile*, *Paramphistomum leydeni*, *Paramphistomum microbothrium*, *Orthocoelium streptocoelium*, *Orthocoelium indonesiense*, *Orthocoelium arambuloi*, *Fischoederius cobboldii*, *Cotylophoron cotylophorum*, *Cotylophoron indicum*, *Calicophoron daubneyi*, *Ceylonocotyle streptocoelium*, *Ceylonocotyle scoliocoelium*, *Ceylonocotyle gigantopharynx*, *Gastrothylax compressus*, *Gastrothylax crumenifer*, *Gastrothylax cobboldii*, *Gastrothylax glandiformis*, *Gastrothylax synethes*, *Carmyerius spatiosus*, *Fischoederius elongatus*, *Fischoederius emiljavieri*, *Velasquezotrema tripurensis*, and *Homalogaster poloniae*. The various distribution of rumen fluke species was observed in cattle and buffaloes in different Asian countries (Figure 5).

Table 1 summarises the reported rumen fluke species by different diagnostic methods. It shows that 61.5% (16/26) reported species of rumen flukes to belong to the family Paramphistomidae, and 38.4% (10/26) belong to the family Gastrothylacidae. Out of 61.5% of family Paramphistomidae, 37.5% (6/16) belong to genus *Paramphistomum* followed by 18.75% (3/16) *Orthocoelium*, 18.75% (3/16) *Ceylonocotyle*, 12.5% (2/16) *Cotylophoron*, 6.25% (1/16) *Fischoederius*, and 6.25% (1/16) *Calicophoron*. Out of 38.4% of the family Gastrothylacidae, 50% (5/10) belong to the genus *Gastrothylax*, followed by 20% (2/10) *Fishoederius*, 10% (1/10) *Velasquezotrema*, 10% (1/10) *Carmyerius*, and 10% (1/10) *Homalogaster*.

The present review shows that most *Paramphistomum* and *Cotylophoron* species have wider distribution and higher prevalence for the reported countries than species of the other genera. *Paramphistomum cervi* has the widest distribution, followed by *Cotylophoron cotylophorum* compared with the other species. Sixteen species; *Paramphistomum gotoi*, *Paramphistomum gracile*, *Paramphistomum leydeni*, *Orthocoelium indonesiense*, *Calicophoron daubneyi*, *Ceylonocotyle streptocoelium*, *Ceylonocotyle scoliocoelium*, *Ceylonocotyle gigantopharynx*, *Cotylophoron indicum*, *Gastrothylax synethes*, *Gastrothylax compressus*, *Gastrothylax glandiformis*, *Fischoederius elongatus*, *Fischoederius emiljavieri*, *Velasquezotrema tripurensis*, and *Homalogaster poloniae* had the narrowest distribution, being reported only in one country each.

Among the reported species of rumen fluke in Asian countries, *Paramphistomum cervi* and *Paramphistomum epiclitum* are the most common species of rumen fluke and the most significant cause of diseases in cattle and buffaloes (Bhatia et al., 2016; Javed Khan et al., 2006; Rafiq et al., 2020). The present review in Asian countries shows the genus of rumen fluke detected by sedimentation method, fluke count

Table 1
Rumen fluke family, species, and their definitive host in different methods reported in Asian countries

Family	Species	Diagnostic methods	Country	Definitive host	References
Paramphistomidae and Gastrothylacidae	*Paramphistomum cervi*, *Cotylophoron cotylophorum*, *Gastrothylax crumenifer*, and *Homalogaster poloniae*	Sedimentation and abattoir	Bangladesh	Cattle and buffalo	Alim et al. (2012); Ara et al. (2021); Azam et al. (2012); Mamun et al. (2011); Saha et al. (2013); T. C. Nath et al. (2016)
Paramphistomidae	*Paramphistomum* spp.	Sedimentation and morphological identification of cercaria	Nepal	Cattle and buffalo	Bista et al. (2018); Regmi et al. (2021)
Paramphistomidae and Gastrothylacidae	*Paramphistomum* spp., *Ceylonocotyle streptocoelium*, *Ceylonocotyle scoliocoelium*, *Ceylonocotyle gigantopharynx, Fischoederius elongatus*, *Gastrothylax synethes*, and *Gastrothylax cobboldii*	Sedimentation and abattoir	Malaysia	Cattle and buffalo	Debbra et al. (2018); Harizt et al. (2021); Khadijah et al. (2017); Schad et al. (1964)
Paramphistomidae and Gastrothylacidae	*Paramphistomum cervi*, *Paramphistomum gotoi*, *Paramphistomum microbothrium*, *Cotylophoron cotylophorum*, *Carmyerius spatiosus*, *Gastrothylax crumenifer*, and *Gastrothylax compressus*	Molecular methods, sedimentation, and abattoir	Iran	Cattle and buffalo	Hajipour et al. (2021); Nikpay et al. (2019); Rafiq et al. (2020)
Paramphistomidae and Gastrothylacidae	*Paramphistomum cervi*, *Paramphistomum epiclitum Cotylophoron cotylophorum*, and *Gastrothylax crumenifer*	Molecular methods, sedimentation, and abattoir	Pakistan	Cattle and buffalo	Ali et al. (2018); Iqbal et al. (2013); Javed Khan et al. (2006); Muhammad et al. (2017); Nazar et al. (2019)
Paramphistomidae and Gastrothylacidae	*Paramphistomum cervi*, *Paramphistomum epiclitum*, *Fischoederius cobboldii*, *Fischoederius elongatus*, *Cotylophoron cotylophorum*, *Cotylophoron indicum*, *Carmyerius spatiosus*, *Gastrothylax crumenifer*, and *Velasquezotrema tripurensis*	Molecular methods, sedimentation, serological and abattoir	India	Cattle and buffalo	Ghatani et al. (2014); Maitra et al. (2014); Malathi et al. (2021); Saifullah et al. (2013); Shameem et al. (2018)

Table 1 (continue)

Family	Species	Diagnostic methods	Country	Definitive host	References
Paramphistomidae and Gastrothylacidae	*Paramphistomum gracile*, *Paramphistomum cervi*, *Paramphistomum epiclitum* and *Fischoederius cobboldii*	Molecular methods, sedimentation, serological test, and abattoir	Thailand	Cattle and buffalo	Anucherngchai et al. (2020); Anuracpreeda et al. (2017); Japa et al. (2020); Jittapalapong et al. (2011); Sanguankiat et al. (2016)
Paramphistomidae and Gastrothylacidae	*Paramphistomum cervi* and *Homalogaster poloniae*	Molecular methods and abattoir	China	Cattle and buffalo	Liu et al. (2009)
Paramphistomidae and Gastrothylacidae	*Cotylophoron cotylophorum*, *Orthocoelium streptocoelium*, and *Homalogaster poloniae*	Molecular method	Japan	Cattle	Itagaki et al. (2003)
Paramphistomidae	*Paramphistomum* spp.	Sedimentation	Cambodia	Cattle	Dorny et al. (2011)
Paramphistomidae and Gastrothylacidae	*Paramphistomum cervi*, *Paramphistomum microbothrium*, and *Gastrothylax crumenifer*	Abattoir	Iraq	Cattle and buffalo	Sadoon Al-Biaty et al. (2011)
Paramphistomidae and Gastrothylacidae	*Paramphistomum* spp. *Orthocoelium indonesiense*, *Orthocoelium arambuloi*, *Fischoederius elongatus*, and *Gastrothylax glandiformis*	Sedimentation and morphological examination	Indonesia	Cattle	Eduardo (2010); Hambal et al. (2020); Rinca et al. (2019)
Paramphistomidae Gastrothylacidae	*Orthocoelium streptocoelium*, *Orthocoelium arambuloi*, and *Fischoederius emiljavieri*	Abattoir	Philippines	Cattle	Eduardo (2010)
Paramphistomidae Gastrothylacidae	*Orthocoelium streptocoelium*, *Orthocoelium arambuloi*, and *Fischoederius emiljavieri*	Abattoir	Philippines	Cattle	Eduardo (2010)
Paramphistomidae and Gastrothylacidae	*Paramphistomum* spp., *Gastrothylax crumenifer*, *Carmyerius* spp., and *Fischoederius elongatus*	Abattoir	Sri Lanka	Cattle and buffalo	Amarasinghe and Kumara (2008)
Paramphistomidae	*Paramphistomum leydeni* and *Calicophoron daubneyi*	Molecular methods and abattoir	Turkey	Cattle	Ozdal et al. (2010); Padak and Karakuş (2021)

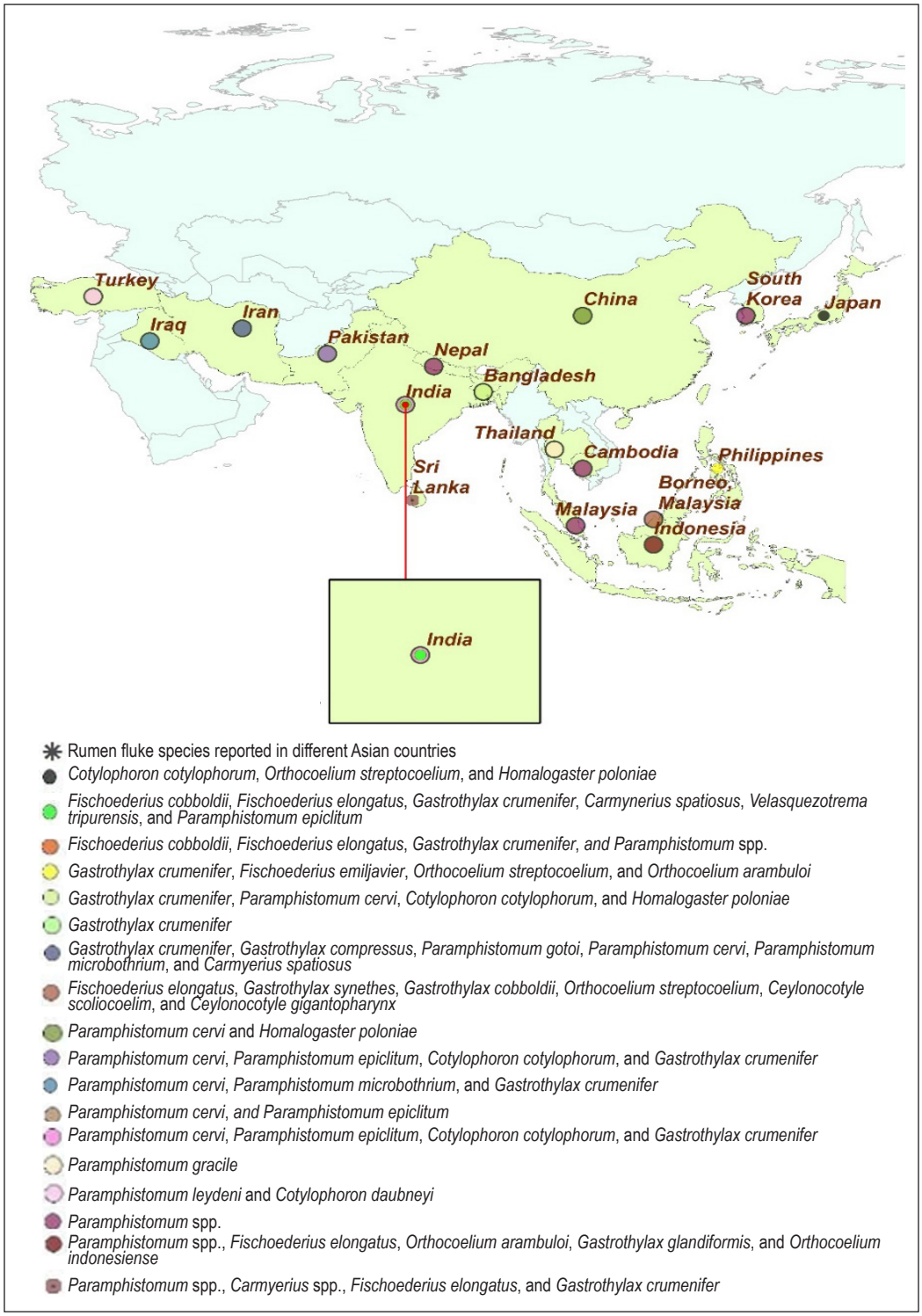

Figure 5. Rumen fluke species reported in cattle and buffaloes of different Asian countries

method from post-mortem examination, and the rumen fluke species detected by serological tests and molecular methods. The sedimentation method may quickly discover eggs in the faeces of an animal carrying an adult fluke. However, this method is of limited value when the disease is in the prepatent period (Taylor et al., 2007), and the eggs cannot be identified up to species level (Mitchell et al., 2021). On the other hand, the filtration method with sieves and sedimentation methods are the most accurate in detecting rumen fluke eggs in faeces (Graham-Brown et al., 2019). Still, it is difficult to identify the rumen fluke species because most have thick-robust bodies and the internal organs are difficult to observe (Lotfy et al., 2010). Molecular methods such as polymerase chain reaction (PCR)-based techniques providing rDNA ITS2 sequence proved to be a good tool for identifying rumen fluke up to species level and determining their phylogenetic relationship (Itagaki et al., 2003; Martinez-Ibeas et al., 2016; Rafiq et al., 2020). The present review in Asian countries shows only *Paramphistomum epiclitum*, *Paramphistomum cervi*, *Paramphistomum leydeni*, *Fischoederius cobboldii*, *Fischoederius elongatus*, *Gastrothylax crumenifer*, *Carmyerius spatiosus*, *Cotylophoron cotylophorum*, *Calicophoron daubneyi*, *Orthocoelium streptocoelium*, *Homalogaster poloniae*, and *Velasquezotrema tripurensis* were detected by molecular and serological methods. However, the rest reported species were detected by sedimentation method and morphological examination of adult flukes.

Epidemiological Factors of Rumen Fluke in Cattle and Buffaloes

The distribution of rumen fluke infecting ruminants has been well documented globally owing to the economic importance from the veterinary standpoint (Huson et al., 2017; Nisar et al., 2021; Pfukenyi & Mukaratirwa, 2018), with a broad geographical spread, particularly in Nigeria, Thailand, and India (Dube, 2010; Kaewnoi et al., 2020; Malathi et al., 2021). Different rumen fluke species predominated in other parts of the world (Rafiq et al., 2020). In areas such as Pakistan and Mexico, *Paramphistomum cervi* is the most frequent species of rumen fluke (Iqbal et al., 2013; Rangel-Ruiz et al., 2003), whereas *Cotylophoron cotylophorum* is the most prevalent species in Australia (Hotessa & Kanko, 2020). *Calicophoron daubneyi* is the most frequent species in the Mediterranean and temperate regions of Algeria, Europe, and the British Isles (Gordon et al., 2013; Huson et al., 2017).

The prevalence and epidemiology of rumen fluke depend on different factors, which include the species of final and intermediate hosts, the fluke potential to infect the hosts, the biological potential of intermediate hosts, and the essential host's management techniques, grazing behaviours, and climate (Horak, 1971; Rangel-Ruiz et al., 2003). Animal grazing area and habitat are significantly associated with the prevalence and intensity of rumen fluke in domestic ruminants (Pfukenyi et al., 2005). Rumen fluke relies on permanent water bodies, such as lakes, rice-growing areas, natural grass pastures with slow-running water, and ponds for their continued

endemicity (Bhatia et al., 2016; Pfukenyi & Mukaratirwa, 2018). In marshy and swampy areas, the rumen fluke outbreak is typical in ruminants and sometimes during the dry season (Pfukenyi & Mukaratirwa, 2018). Cattle develop good immunity, and outbreaks are mainly confined to the animals. However, adults continue to carry low levels of adult parasites and serve as an important reservoir of infection for snails (Taylor et al., 2007).

Snail Intermediate Host

Snail-borne parasite diseases, such as trematodes, pose a serious health risk to humans and animals in many tropical and subtropical areas, as well as causing substantial economic concerns (Lotfy et al., 2010; Lu et al., 2018). Most freshwater/mud snails are the obligatory intermediate host for at least 71 trematode parasites (Bargues & Mas-Coma, 2005). The intermediate host of rumen flukes is freshwater/mud snails such as *Planorbis*, *Lymnaea*, and *Bulinus* (Iqbal et al., 2013; Javed Khan et al., 2006; Taylor et al., 2007). Lymnaeid snails, the intermediate host of rumen fluke, play an essential role in the epidemiology of rumen fluke (Bargues & Mas-Coma, 2005).

The common species of snails that can act as intermediate hosts of rumen fluke reported from different Asian countries include *Radix rubiginosa*, *Radix auricularia*, *Lymnaea truncatula*, *Lymnaea stagnalis*, *Lymnaea natalensis*, *Lymnaea palustris*, *Lymnaea viridis*, *Lymnaea luteola*, *Bulinus truncates*, *Bulinus furskalii*, and *Indoplanorbis exustus* (Dodangeh et al., 2019; Dung et al., 2013; Martin & Cabrera, 2018; Mohammed et al., 2016). Therefore, rumen fluke is found at high prevalence in most tropical and sub-tropical countries such as Bangladesh, Thailand, Indonesia, and India (Azam et al., 2012; Kaewnoi et al., 2020; Hambal et al., 2020; Malathi et al., 2021), particularly in locations where the climatic and environmental conditions are favourable for the intermediate host snail's survival and multiplications (Gordon et al., 2013; Taylor et al., 2007). Also, these intermediate hosts play an essential role in completing the life cycle of rumen fluke and transmitting the disease (Lotfy et al., 2010; Pfukenyi & Mukaratirwa, 2018).

Furthermore, livestock movement, transport, and export/import have proved their ability to passively proliferate lymnaeid snails (Bargues & Mas-Coma, 2005). The situation is complicated by the ability of the snails to aestivate on dry pastures and become reactivated on the return of rainfall in some areas (Mas-Coma et al., 2009). In addition, moisture and irrigated pastures are often sufficient for the survival of the intermediate host (Bhatia et al., 2016).

Table 2 shows data on the prevalence of rumen fluke infection from 17 countries. The prevalence data of rumen flukes are based on the fluke count and sedimentation method and are primarily conducted in cattle. In Asian countries, species-specific for prevalence data are limited due to problems in identifying rumen fluke species. In the present review, 52 studies used the sedimentation method, 15 fluke count (abattoir inspections), 8 molecular methods, and 2 used serological tests. The fluke

Table 2
Prevalence of rumen fluke in cattle and buffaloes in Asian countries based on faecal examination and fluke count

Host	Country	Total animals	Ranging prevalence (%)	References
		Faecal examination		
Cattle	India	12,554	0.8–30.52	A. Gupta et al. (2013); G. Das et al. (2018b); Krishna Murthy and D'Souza (2016); M. Das et al. (2018); Malathi et al. (2021); Maitra et al. (2014); Preethi et al. (2020); S. Nath et al., (2016); Sreedhar (2009); Thakre et al. (2019)
Cattle	Pakistan	1,279	3.65–8.75	Muhammad et al. (2017); Nisar et al. (2021)
Cattle	Iran	1,000	19.5	Hajipour et al. (2021)
Cattle	Thailand	4,402	10.2–97.17	Japa et al. (2020); Jittapalapong et al. (2011); Income et al. (2021); Kaewnoi et al. (2020); Thanasuwan et al. (2021); Yuwajita et al. (2014)
Cattle	Indonesia	303	4–81	Dwinata et al. (2018); Hambal et al. (2020); Rinca et al. (2019)
Cattle	Bangladesh	1,738	18.3–70.8	Akanda et al. (2014); Hassan et al. (2020); Paul et al. (2012); Rashid et al. (2015); Saha et al. (2013)
Cattle	Nepal	165	36.71–39.5	Bista et al. (2018); Regmi et al. (2021)
Cattle	Sri Lanka	147	1.36	Gunathilaka et al. (2018)
Cattle	Malaysia	219	8	Khadijah et al. (2017)
Cattle	Taiwan	310	8.7	Tung et al. (2012)
	Total	22,117	0.8–97.17	
Buffalo	Cambodia	2,391	45	Dorny et al. (2011)
Buffalo	Vietnam	334	78	Geurden et al. (2008)
Buffalo	India	11,943	08.6–46.97	A. Gupta et al. (2018); G. Das et al. (2018a, 2018b); Krishna Murthy and D'Souza (2016); M. Das et al. (2018); Maharana et al. (2016); Malathi et al. (2021); S. Nath et al. (2015, 2016); Swarnakar et al. (2015)
Buffalo	Pakistan	438	12.8–12.9	Muhammad et al. (2017); Nazar et al. (2019)
Buffalo	Nepal	43	21.4	Bista et al. (2018)
Buffalo	Malaysia	129	75.2	Harizt et al. (2021)
Buffalo	Sri Lanka	16	18.75	Gunathilaka et al. (2018)
Buffalo	Bangladesh	1,319	15–78.4	Ara et al. (2021); Mamun et al. (2011); Roy et al. (2016); Saha et al. (2013); T. C. Nath et al. (2016)
	Total	16,613	08.6–78.4	
		Fluke count		
Cattle	Iran	2,406	19.7–36.9	Hajipour et al. (2021); Khedri et al. (2015); Nikpay et al. (2019)
Cattle	Bangladesh	612	20.13–90.6	Alim et al. (2012); Azam et al. (2012)

Table 2 *(continue)*

Host	Country	Total animals	Ranging prevalence (%)	References
Cattle	Iraq	518	6.45–60	Kurtpinar and Latif (1970); Sadoon Al-Biaty et al. (2011)
Cattle	Turkey	447	8.95	Ozdal et al. (2010)
Cattle	Pakistan	34	17.64–50.7	Raza et al. (2009)
Cattle	South Korea	2,124	60	Kang and Kim (1988)
	Total	6,141	6.45–90.6	
Buffalo	India	1,619	4.29–74.7	Patil et al. (2012); Swarnakar et al. (2014)
Buffalo	Pakistan	3,189	17.3–75.07	Asif Raza et al. (2012); Iqbal et al. (2013); Javed Khan et al. (2006)
Buffalo	Iraq	146	40	Kutpinar and Latif (1970)
	Total	4,954	4.29–75.07	

count method is suitable for detecting rumen fluke in slaughtered animals, but the sedimentation method is the most reliable and cheap method to detect rumen fluke eggs in live animals (Graham-Brown et al., 2019; Taylor et al., 2007).

In Table 2, the coprological prevalence of faecal examination varies from 0.8% to 98.17% and 0.86% to 78.4% in cattle and buffaloes, respectively. The prevalence of rumen fluke by fluke counts method range between 6.45% to 90.6% and 4.29% to 75.07% in cattle and buffaloes, respectively. This prevalence of rumen fluke is variable in different countries, possibly due to climatic conditions of temperature and rainfall, different sample sizes, and livestock management systems (González-Warleta et al., 2013; Pfukenyi et al., 2005). Table 2 also shows that the prevalence of rumen fluke in Asian countries is higher in cattle than in buffaloes. The higher prevalence in cattle contributed to a greater sample size due to the high cattle population compared to buffaloes.

Pathogenesis and Clinical Sign in Cattle and Buffaloes

Pathological changes related to natural paramphistomosis have not been studied (Fuertes et al., 2015). Studies showed that immature rumen fluke is pathogenic and causes catarrhal to necrotic inflammation with thickening and ulceration in the small intestine after nine days of exposure to infected pasture, which cause productivity loss such as decreased in meat and milk, low nutrient conversation, weight loss, and fertility reduction (Chaudhry et al., 2017; Malrait et al., 2015; Mohanta et al., 2017). The frequency and significance of lesions caused by the adult rumen fluke are unclear (Toledo et al., 2006). Most authors suggested that the adult stage of rumen fluke does not produce any pathogenic effect and is harmless, but some experimental studies have shown that in heavy infection, it is associated with ruminal papillae atrophy and ulceration at the site of fluke attachment (Fuertes et al., 2015; Mohanta et al., 2017). The ruminal atrium of cattle

with natural paramphistomosis had the highest parasite burden. According to a post-mortem investigation of the forestomach, the *Calicophoron daubneyi* was the only species of the Paramphistomidae family identified in these animals (González-Warleta et al., 2013). Also, it has been proved that young animals under two years old are the most vulnerable, as adults build immunity more quickly and for more extended periods (Sanabria & Romero, 2008).

Variable mucosal oedema and villous atrophy are seen on histopathological examination of the affected intestine, including concomitant hyperplasia of mucosal crypts and submucosal Brunner's glands and infiltration of the mucosa and submucosa by lymphocyte, eosinophils, mast cells, plasma cells, and globule leucocytes (Fuertes et al., 2015). Mucosa-associated lymphoid follicles can occur on rare occasions (N. K. Gupta, 1993). Submucosal Brunner's glands isolated from the luminal environment may provide an ideal environment for the early growth of immature fluke (Fuertes et al., 2015). The hyperplastic changes persist until the rumen fluke migrates to the rumen, and it is possible to distinguish the microscopic differences from other endoparasites (Fenemore et al., 2021; Huson et al., 2017; N. K. Gupta, 1993).

The clinical signs of rumen fluke which have been reported from different countries include dullness, diarrhoea, which is accompanied by anorexia and intense thirst, weight loss, depression, severe water scour, anaemia, hypoproteinaemia, hypalbuminaemia, sub-mandibular oedema, and rectal haemorrhage (Fenemore et al., 2021; Lotfy et al., 2010; Rangel-Ruiz et al., 2003). Mortality in an acute outbreak can be as high as 90% (Dube, 2010; Taylor et al., 2007).

Diagnosis

Rumen fluke diagnosis includes clinical signs, egg detection in the faecal sample, post-mortem examination, and molecular methods (Malrait et al., 2015; Rieu et al., 2007). Commercial tests like Flukefinder and FLOTAC are also available (Cringoli et al., 2010). Commercial tests are more sensitive than sedimentation methods (Duthaler et al., 2010). Unlike liver fluke, no commercial immunological diagnostic test is available for rumen fluke. Clinical signs can diagnose diseases in live animals (Taylor et al., 2007). Laboratory testing may quickly discover eggs in the faeces of an animal carrying an adult fluke. Still, this method is of little value since the disease occurs during the prepatent period, and the eggs could not be identified up to species level (Fenemore et al., 2021). Among the coprological tests, the filtration method with sieves and sedimentation methods are the most accurate for detecting rumen fluke eggs in faeces (Horak, 1971).

In post-mortem examination, adult fluke can be detected easily. Still, it is difficult to identify at the species level because challenging to observe the thick, robust bodies, and internal organs (Lotfy et al., 2010). A faecal examination is the best method for detecting the parasite in

live animals and determining the burden of fluke in the forestomach of live animals (Sargison et al., 2016). The fluke count method is a reliable method with good sensitivity and specificity compared to faecal egg count, and it has been proven that more than 100 eggs per gram indicate the presence of more than 100 adult rumen flukes in the rumen or reticulum (Rieu et al., 2007; Sargison et al., 2016). On the other hand, immunological diagnosis is still early for diagnosing rumen fluke (Tandon et al., 2014). However, a few studies proved that coproantigen detection provides an excellent alternative to the conventional method for diagnosing rumen fluke in livestock (Anuracpreeda et al., 2017; Saifullah et al., 2013). Finally, understanding the rumen fluke life cycle and control options requires accurate species identification for rumen fluke (Gordon et al., 2013). Because of all these problems, a molecular method such as PCR-based techniques providing rDNA ITS2 sequence proved to be good tools for identifying rumen fluke up to species level and determining their phylogenetic relationship (Chamuah et al., 2016; Itagaki et al., 2003; Martinez-Ibeas et al., 2016).

Treatment and Control of Rumen Fluke

The typical flukicides used in cattle and sheep do not kill rumen fluke (Animal Health and Veterinary Laboratories Agency [AHVLA], 2013). Among antihelmintic drugs, oxyclozanide and closantel with faecal egg count reduction (FECR) values of 97% to 99% were found to be the drug of choice for the treatment of rumen fluke (Arias et al., 2013; Fenemore et al., 2021). Mild infections do not affect animal health or productivity (Fenemore et al., 2021). According to Animal Health Ireland (AHI) (2011), detecting rumen fluke eggs in faecal samples or detecting adults in small numbers in the rumen is not a reason to carry out specific control measures (AHI, 2011). Current veterinary advice is necessary to avoid the overuse of any flukicide to reduce resistance. Roughly treating rumen fluke is rarely justified, except on farms where severe disease and economic losses are confirmed (AHI, 2011).

The disease incidence is closely linked to environmental conditions, ecology, and the infection of intermediate snail hosts in a given location (Tariq et al., 2008). High rainfall areas and areas where animals have access to streams, ditches, ponds, wetlands, and marshy regions have a greater prevalence of rumen fluke (Fenemore et al., 2021). Keeping domestic ruminants away from infected pastures is the way to avoid rumen fluke infections (Pfukenyi et al., 2005). The most effective strategy for controlling rumen fluke is to remove the snail intermediate host from the rumen fluke life cycle (N. K. Gupta, 1993). It is recommended that wetlands or marshy/ swampy areas be fenced off or drained and ensure that clean pastures and cercaria-free troughs are provided to the livestock (Taylor et al., 2007).

CONCLUSION

In the present review, 26 rumen fluke species belonging to two families

occurred in cattle and buffaloes in Asian countries. The prevalence of rumen fluke is high in some Asian countries. Genus *Paramphistomum* and *Cotylophoron* in the family of Paramphistomidae have a wider distribution and higher prevalence than the species of other genera. The prevalence data show that the fluke count method is reliable for determining the prevalence of rumen fluke in slaughtered cattle and buffaloes. In contrast, the sedimentation method is highly suitable for detecting rumen fluke infection in live animals. Molecular methods proved good tools for identifying rumen fluke up to species level. Based on recent studies, rumen fluke should now be considered a vital production disease of ruminant livestock in Asia. It is necessary to comprehend and manage all aspects of epidemiological factors that may impact cattle and buffaloes' productivity and farming efficiency to control the rumen fluke infections. Therefore, more studies are needed to identify the species of rumen fluke in most Asian countries, explore the economic impact of rumen fluke, detect a specific type of snail that acts as an intermediate host for rumen fluke, the anthelmintic efficacy, and develop the diagnostic techniques that can detect the prepatent infections of rumen fluke in livestock.

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Growth Analysis of Lettuce (*Lactuca Sativa* L.) Using Nutrient Film Technique (NFT) in Hydroponic Systems

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ABSTRACT

This study aims to determine the effects of planting media and gutter slopes on the growth of lettuce (*Lactuca sativa* L.) using the Nutrient Film Technique (NFT) in a hydroponic system. This research was conducted at the Experimental Garden of the Faculty of Agriculture, Universitas Lakidende Unaaha, Konawe Regency, Indonesia, in October 2021. The two treatments were arranged in a randomized block design (RBD) and repeated in three replications. The first treatment consisted of two types of growing media: sponge medium (M1) and rockwool medium (M2). The second treatment consisted of two kinds, i.e., a 3% gutter slope of pipes (K1) and a 5% gutter slope of pipes (K2). The observed variables were plant height, number of leaves, and plant fresh weight. All collected data were analyzed using analysis of variance (ANOVA) followed by the least significant difference (LSD) test at a 5% level. This study has revealed three major results. Firstly, the interaction between planting media and the slope of the gutter pipes significantly affects plant height and the fresh weight of lettuce. Secondly, the planting media or the slope of the gutter pipes independently has a significant effect on plant height, several leaves, and the fresh weight of lettuce plants. Lastly, the treatment of rockwool planting media and a 5% slope of gutter pipes significantly improved the lettuce growth.

Keywords: Pipes, rockwool, slope, sponge, yield

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INTRODUCTION

Hydroponics is the latest model of plant maintenance that does not use soil, water, and porous material media, such as burnt husks, fine sand, sponge, or rockwool. Therefore, nutrient adequacy is fulfilled

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from the nutrient solution from the watering. Agricultural cultivation technology using a hydroponic system is a second option for people with limited land or yards, who want to grow beneficial plants (Mavianti & Irawan, 2021).

Plant maintenance through a hydroponic system requires support by using vehicles that can increase plant growth and development. These supporting vehicles have roles integrated with hydroponic technology, including greenhouses, irrigation, planting media, managers, seeds, land management, and nursery systems. The primary goal of farming using a hydroponic method is commercial. Moreover, a hydroponic method does not require a large area of land but can significantly work in front or side of the house and yard.

The nutrient film technique (NFT) is hydroponic that is extensively utilized in the production of vegetables such as lettuce. Plant roots are placed in a thin layer of nutrient solution in a hydroponic technique. This system is an example of plant preservation. The nutrient solution is circulated and has nutritional content considering the needs of the plants. The root system can grow on a mixture of nutrients. Moreover, several factors must be considered, such as when excessive water will occur and factors decreasing the amount of oxygen. The nutrient content of the NFT method is specially designed, with a maximum solution height of 3 mm, so that water, nutrients, and oxygen needs can be met.

The NFT is one of the hydroponic water culture techniques that allow plants to receive nutrients and water by circulation in a shallow and sloped layer. It is also a planting technique that grows lettuce on paragon pipes with a 1–5% slope. This system does not need growing media because the roots of plants are submerged in a thin layer of nutrients circulated and regulated using a timer. The NFT (Figure 1) has been widely used to produce vegetable crops, such as curly lettuce (Eprianda et al., 2017).

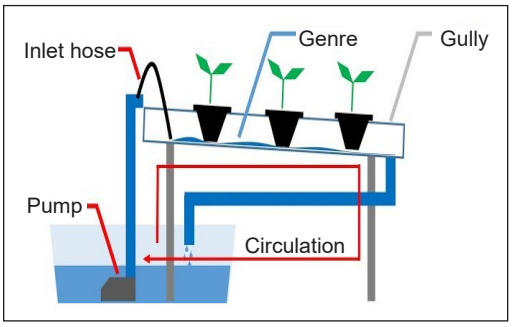

Figure 1. Nutrient Film Technique (NFT) of the hydroponics system scheme

Another technique of planting is using a wick system applied to some flannel in a capillary action of nutrient solution, which is a passive way (Silviana et al., 2020). Suaib et al. (2020) have grown tomato plants in a wick system and studied the effects of aluminum on the plants' growth and production using a hydroponic wick system. Lettuce (*Lactuca sativa* L.) is a plant that belongs to the Compositae family and can grow in cold and tropical areas. Lettuce marketing is getting higher along with economic and population growth (Siregar et al., 2015).

Lettuce is one of the leaf vegetable plants generally consumed in raw material. It is a horticultural plant with good nutritional content for humans (Kim et al., 2016). Moreover, it has a relatively high nutritional content and contains high-quality values, such as nutrition, color, aroma, taste, and texture. This research observed the NFT system to plant lettuce and examined the combination between several planting media and slopes of gutter pipes as a treatment.

Research on hydroponics with the nutrient film technique (NFT) method in plant cultivation, especially vegetables, has been carried out. However, no study has investigated the combination of sponge and rockwool growing media with a 5% slope of gutter pipes, especially on lettuce plants. Sponge and rockwool plants media combined with a 5% slope of gutter pipes are one of the new and strategic studies to support sustainable vegetable production and meet national nutritional needs. Therefore, this study aims to determine the effect of sponge and rockwool growing media combined with the slope of the gutter pipe on the growth and yield of lettuce.

METHODS

This experimental study was conducted at the Experimental Garden of the Faculty of Agriculture, Universitas Lakidende Unaaha, Konawe Regency Southeast Sulawesi, Indonesia, in October 2021. The materials used in this study were lettuce seeds, planting media (sponge and rockwool), plant nutrients (AB MIX fertilizer, Indonesia), and water. Meanwhile, the tools used were a ruler, line meter, aerator (aquarium pump), total dissolved solids (TDS) (metal measuring device of dissolved in water), pH meter, 7 mm polyethylene (PE) hose, 7 mm seal, 250-liter capacity water reservoir, 4-meter-long gutter, inch pipe, drill, grinder, Korean hacksaw glue, and ultraviolet (UV) plastic.

Furthermore, the study employed a randomized block design (RBD) and two treatments: sponge and rockwool planting media, as well as 3 and 5% of gutters. Each treatment was repeated three times. Thus, four treatment combinations and three repetitions were obtained. The experimental units were $4 \times 3 = 12$ with 12 samples. Randomization was carried out directly on 12 experimental units.

Variable measurement was done in non-destructive and destructive ways. Non-destructive measurements were carried out once a week or one month after planting. Plant height (cm) was measured from the base of the stem to the tip of the highest leaf using a ruler. Observations were conducted for a week. The number of leaves was observed every seven days for a month. This study only measured perfectly formed leaves that contain chlorophylls and could photosynthesize. Destructive observations on another side were carried out at the end of the observation with the variable of fresh weight (g). The observation was carried out by weighting all parts of the fresh plants directly after the harvesting. All data collected were analyzed by ANOVA followed by the least significant difference (LSD) test at a 5% significance level.

RESULTS

Plant Height

The finding of the variance analysis indicates that the planting material and the slopes of the gutter pipes have an interaction. Independent treatment of planting media and the slope of the gutter pipes affects the plant height (cm). The best treatment was the combination of the M2K2 (rockwool medium with gutter slope) as shown in Table 1. The average difference of treatment combinations on plant height is presented in Table 1.

Table 1
Effects of planting media and gutter slopes of pipes on plant height (cm)

Planting media	Gutter slopes of pipes (%)		Average scores
	K1(3%)	K2 (5%)	
M1 (Sponge)	13.67[a]	16.30[b]	14.98[a]
M2 (Rockwool)	15.57[b]	16.40[b]	15.99[b]
Average scores	14.62[a]	16.35[b]	
LSD 0.05 = 0.96			

Note. Numbers followed by different letters in the same rows or columns show a significant difference

Number of Leaves

The variance analysis has revealed that the treatment of planting media and the slope of the gutter pipes have no interaction effects. However, the number of leaves is significantly influenced independently by planting media treatment or the level of the gutter's slope. The best treatment in Table 2 was the combination of the M1K2 treatment (sponge medium with 5% gutter slope). Table 2 shows the results of the mean difference test evaluating the influence of the treatment on the number of shoots.

Table 2
Effects of planting media and gutter slopes of pipes on the number of leaves

Planting media	Gutter slopes of pipes (%)		Average scores
	K1(3%)	K2 (5%)	
M1 (Sponge)	6.33[a]	7.33[b]	6.83[a]
M2 (Rockwool)	5.67[a]	6.00[a]	5.84[b]
Average scores	6.00[a]	6.67[b]	
LSD 0.05 = 0.66			

Note. Numbers followed by different letters in the same rows or columns indicate a significant difference

The highest number of leaves is affected by the 5% slope treatment (K2). Meanwhile, the treatment of the growing media has the highest number of leaves in the treatment using a sponge (M1) growing medium.

Leaf Fresh Weight

The variance analysis has revealed that planting media treatment and the gutter pipe slope significantly affect the fresh leaf weight. The best treatment in Table 3 was the combination of the M2K2 treatment (rockwool medium with 5% gutter slope. The results of the mean difference are presented in Table 3.

Table 3
Effects of planting media and gutter slopes of pipes on fresh leaf weight (g)

Planting media	Gutter slopes of pipes (%)		Average scores
	K1(3%)	K2 (5%)	
M1 (Sponge)	203.33[a]	283.33[c]	234.33[a]
M2 (Rockwool)	250.00[b]	288.33[c]	269.17[b]
Average scores	226.67[a]	285.83[b]	
LSD 0.05= 20.78			

Note. Numbers followed by different letters in the same rows or columns show a significant difference

Table 3 describes that the treatment of rockwool (M2) planting medium and 5% gutter slope of pipe (K2) produce the highest fresh leaf weight of lettuce because the rockwool has a much higher porosity than the sponge medium.

DISCUSSION

This experiment has revealed that the planting media and the slopes of the gutter pipe affect the growth of lettuce plant height because the plants could bind some water and nutrients. The ability to bind water and nutrients in a medium depends on the particle sizes, shapes, and porosity of the medium. The smaller the particle size, the larger the pore surface area, and the greater the media's ability to absorb and hold some water. Porous media also have a greater ability to hold water. Sponge media have a much higher porosity than rockwool media. Thus, the effects of planting media (sponge and rockwool) on lettuce's growth in this study are in line with those on certain plants' growth (Bachtiar et al., 2017; Perwitasari et al., 2012; Suryani et al., 2017). A good planting medium can give enough water and nutrients for plant growth. It may be established in soil with good air and water management, solid aggregates, good water holding capacity, and sufficient root space (Lim et al., 2021). The results of this research conclude that lettuce plants growing on rockwool medium with a slope of 5% has fast growth.

In addition, the nutrient film technique is a plant cultivation system that circulates plant roots in a thin stream of water and contains elements required by plants. The root system in the NFT is submerged in the nutrient solution in only a certain part of the root layer. Meanwhile, the aeration of the root zone depends on several factors, such as pore space, medium particle sizes, and container height. The container height (pot) affects the ratio between water and air in root media (Tai et al., 2014). Therefore, growing media is one of the crucial factors in hydroponics. Besides soil media, hydroponic planting can use other media, such as water. The most important requirements for hydroponic media are light and porous. Since each medium has a different weight and porosity, it should be determined by taking into account the lightest and the best porosity (Primatoro & Yovita, 2005).

The number of leaves formed is different because it is related to the reduced amount of carbohydrates in the leaves with the porosity of the growing media. Due to cell division, elongation, and differentiation, lettuce leaves more significantly grow on sponge medium. In this condition, carbohydrate is needed (Maulana et al., 2020; Rahardjo et al., 2013).

According to Sari (2013), the slope of the pipe in a hydroponic system will impact the growth and yield of a plant. The slope, in this case, will affect leaf production, width, plant height, and root length.

Porosity is the proportion of medium pore space in a medium volume occupied by water and air, and it measures drainage and soil aeration. A porous medium has adequate pore space to allow water and

air to move more flexibly. It reported that planting medium with high porosity could increase the mung bean's lateral root length and shoot its dry weight (Kusuma et al., 2013; Moschou et al., 2022; Thalib, 2019). Increasing soil porosity can increase plant growth (Anastasia et al., 2014). A good hydroponic planting medium is a medium that has good porosity so that water and air can circulate well around the plant root system (Barus et al., 2021; Gui et al., 2010; Titouna & Bougoul, 2013). It is no surprise that rockwool's porous structure helps lettuce plants grow faster than they would in a sponge medium.

The slope of the pipe or pipe gutter in a hydroponic system influences the plant's growth and yield. The slope, in this case, will affect the production of whole leaves, leaf width, plant height, and root length. This influence can reach up to 5% (Sari, 2013).

The slope of the gutter is one factor that increases plant production. It has been shown that the best treatment was the M2K2 treatment combination (rockwool medium with 5% gutter slope) in Tables 1 and 3, while the M1K2 treatment combination (sponge medium with 5% gutter slope) was in Table 2. Simbolon (2011), as well as Surtinah and Lidar (2018), have revealed that the slope of the gutter affects plant growth and production in terms of root length, fresh root weight, dry root weight, number of leaves, leaf areas, plant height, crown diameters, bulb diameters, fresh crown weight, and canopy dry weight. This diversity occurs because the gutter's slope affects the roots' ability to absorb nutrients and the thickness of nutrient layers. Too thin layers will hamper roots from absorbing nutrients; in contrast, too thick layers will hamper roots from absorbing nutrients; too thick layers will hamper plants from breathing (Asmana et al., 2017).

The different responses to plant growth in the two treatment factors are visible in the fresh leaf weight, as presented in Figures 2 and 3.

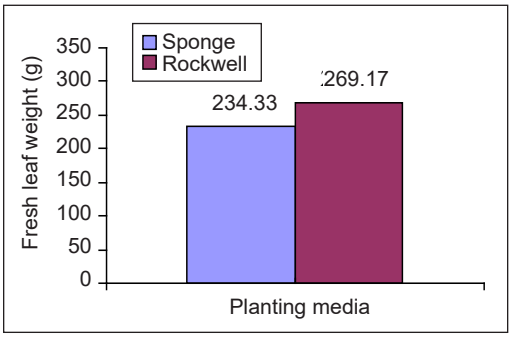

Figure 2. Fresh leaf weight of lettuce plants on different types of planting media treatment

Figure 3. Fresh leaf weight of lettuce plants on pipe gutter slope treatment

CONCLUSION

The study revealed three major factors that could affect the growth of lettuce plants using NFT in hydroponic systems. First, the interaction between planting media and

the slope of the gutter pipes significantly affects plant height and the fresh weight of lettuce. Second, the planting media or the slope of the gutter pipes independently has a significant effect on plant height, several leaves, and the fresh weight of lettuce plants. Third, the treatment of rockwool planting media and gutter pipe slope 5% (M2K2) is the best and is more real in growing lettuce.

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Incidence of Food Poisoning Outbreaks in Pahang, Malaysia, for Six-Year, from 2013 to 2018

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ABSTRACT

The Food Safety and Quality Division (FSQD) in Malaysia is the competent authority tasked with ensuring food safety throughout the food supply chain within the country. Despite implementing various regulations toward improving food hygiene standards in Malaysia, outbreaks of food poisoning cases continued to occur in Malaysia. This cross-sectional study was designed to explore the occurrence of food poisoning incidents in Malaysia, within the Pahang state, from 2013 to 2018 via both reported passive case detection (PCD) and active case detection (ACD) food poisoning incidents. Upon detecting all the food poisoning cases using both PCD and ACD, the people identified to have suffered from food poisoning underwent a structured interview for investigators to elicit all relevant information about the food poisoning incident. Results showed that in Pahang, the number of reported episodes fluctuated from 2013 until 2018, with an average of 21 food poisoning episodes occurring yearly, reaching a maximum in August and a minimum in May. Furthermore, Kuantan, being the state capital, had reported an exceptionally high total number of reported incidents of food

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poisoning with a total of 48 episodes over six years from 2013 to 2018, while Kuala Lipis had only one incident reported during the same period (which was reported in 2016). Finally, this study concluded that adequate measures must always be taken to minimise the occurrence of food poisoning, especially when preparing foods in large quantities.

Keywords: Foodborne diseases, foodborne illnesses, food poisoning, Malaysia, Pahang

INTRODUCTION

Food poisoning often results from consuming any food or water which is contaminated. These contaminants are usually bacteria and/or their toxins, parasites, viruses, or chemicals. It can be infectious or non-infectious. Infectious food poisoning occurs after consuming food or water contaminated by microorganisms (including bacteria, viruses, and parasites) or their toxins. In contrast, non-infectious food poisoning occurs after consuming any food contaminated with chemicals or toxins. A food poisoning outbreak is said to have occurred whenever there are at least two cases of a similar foodborne disease (FBD) that have arisen from different people consuming a common food together (World Health Organization [WHO], 2008). The symptoms of food poisoning can often have varying degrees of symptoms, which include abdominal pain, vomiting, diarrhoea, and headache. Depending on its causative agent, food poisoning episodes will usually last from a few hours to several days after an oral intake of contaminated food or fluid. Serious cases may result in severe, life-threatening complications involving liver, kidney, and nerve functions leading to permanent disability or death. Mild food poisoning episodes are usually self-limiting, and patients will recover without receiving any specific treatment; however, severe forms of food poisoning may require antibiotic treatment and hydration within a hospital setting (Pardal et al., 2020).

Human hands contribute the most to food poisoning incidents as they are always in contact with the ambient environment; and the ubiquitous nature of pathogenic food microorganisms can often be transmitted from the hands to reach the mucous membranes throughout the body (Hawker et al., 2012), including those found in the mouth, nose, and eyes. Common pathogens responsible for most food poisoning cases are norovirus, *Campylobacter* spp., enterotoxigenic *Escherichia coli*, diarrheal disease due to non-typhoid *Salmonella enterica*, and *Shigella* spp. (Kirk et al., 2015); most hospitalisations have resulted from food poisoning episodes caused by *Salmonella* spp., norovirus, *Campylobacter* spp., *Toxoplasma gondii*, and *E. coli*. It is noteworthy that food poisoning caused by *Salmonella* spp., *Toxoplasma gondii*, *Listeria monocytogenes*, norovirus, and *Campylobacter* spp. can occasionally be fatal. Besides, it is a worrying trend for the reported incidence of food poisoning globally to increase yearly (Abdul-Mutalib et al., 2015). According to World Health Organization (WHO, 2015), contaminated

food has resulted in more than 50% of diarrhoea cases worldwide, causing 550 million sick people and 230,000 deaths, together with 125,000 deaths in young children below five years old. Data obtained from several studies reported worldwide have shown that there are already 48 million people suffering from food poisoning annually, which results in 128,000 hospitalisations and 3000 deaths within the USA (Centers for Disease Control and Prevention [CDC], 2018). Developing countries have the most recorded number of food poisoning cases and fatalities (Elshafie, 2017). Globally, a high incidence and fatality rate of foodborne diseases is reported within Africa and Southeast Asia (WHO, 2015). The Foodborne Disease Burden Epidemiology Reference Group (FERG) of the WHO reported that the Southeast Asia region has the second-highest reported foodborne illnesses globally (Kirk et al., 2015). It was widely reported worldwide that cross-contamination due to the unhygienic handling of raw meat (Ansari-Lari et al., 2010) caused most food poisoning cases in many developing countries.

Likewise, in Malaysia, food poisoning is a perennial health problem. Its incidence rate was 44.18/per 100,000 population in 2010, which then increased to 50.42/per 100,000 in 2014 and decreased slightly to 47.2/per 100,000 in 2016. In Malaysia, the fatality rate of food poisoning was approximately 0.041/per 100,000 population in 2016 (Ministry of Health [MOH], 2016). Malaysia's high incidence of food poisoning is partly attributed to its climate conditions and typical Malaysian cuisine features. The hot and humid climate conditions in Malaysia and the combination of various raw ingredients in a typical Malaysian cuisine can enhance the likelihood of food contamination and spoilage, which often result in an increased incidence of food poisoning in Malaysia. Therefore, this study was designed to identify those factors that may play a specific role in contributing to food poisoning in Malaysia, especially in Pahang. To this end, the study team made the necessary arrangements for this cross-sectional study to be conducted simultaneously with routine surveillance of all the reported food poisoning incidents within Pahang from 2013 to 2018 because previous data provided by the Malaysian Ministry of Health (MOH) in 2014 had reported that incidence of food poisoning in Malaysia decreased in 2012 but increased slightly in 2013 (Abdul-Mutalib et al., 2015), which had also shown to increase mortality in Malaysia (Abdul-Mutalib et al., 2015).

Pahang is the largest state by area within Peninsular Malaysia, and its total land area is 35,965 km^2, with a total population of 1.68 million people (Department of Statistics Malaysia [DOSM], 2021); it consists of a total of 11 districts. In addition, it usually experiences two monsoon seasons per year. Therefore, certain low-lying areas within the Pahang state have frequently experienced flooding, which might result in water supplies and food ingredients contamination in these areas. This investigation aimed to verify each suspected outbreak's cause, determine

its magnitude identify possible causes, and implement necessary control and preventive measures. Thus, findings from this study can be utilised by stakeholders to introduce effective and/or novel interventions for mitigating the risk of food poisoning and for safeguarding consumer health by strengthening food control; as well as FBD surveillance systems (including those for food poisoning) to reduce the burden of food poisoning within Malaysia.

MATERIALS AND METHODS

Study Design and Population

It is a retrospective, observational, and quantitative study involving collecting all relevant data on the incidence of food poisoning within all the 11 districts of Pahang, including Bentong, Bera, Cameron Highlands, Jerantut, Kuantan, Lipis, Maran, Pekan, Raub, Rompin, and Temerloh. The source populations for this study provide all the reported cases of food poisoning outbreaks in Pahang from 2013 to 2018, which are based on data collected by the food poisoning outbreak report FWBD/KRM/BG 001 (2006 amendments).

Source of Data and Data Collection Method

This study applied passive case detection (PCD) and active case detection (ACD) to report food poisoning incidents in Pahang state via routine surveillance within all the 11 districts of Pahang from 2013 to 2018. For this study, a case of food poisoning is defined as persons within the state of Pahang who had presented with abdominal pain, vomiting, and/or diarrhoea from 2013 to 2018 and who also consumed local food.

Typical laboratory-based surveillance is "passive", in that detecting food poisoning incidents only relies on the laboratories to report them to public health authorities (WHO, 2008). However, more often, such passive surveillance for case detection may not be adequate; particularly when there is suspicion of the occurrence of food poisoning; hence, "active" surveillance is recommended for a pre-determined duration during an outbreak to supplement the findings obtained from passive surveillance alone (WHO, 2008). Under such circumstances, the food safety or public health authorities will regularly contact the laboratories to constantly seek updates about recent positive tests indicative of potential FBDs (WHO, 2008).

The same principles above shall apply to this study. While PCD represents typical routine surveillance that is "passive", which relies totally on the sources of food poisoning incidents to report them to public health authorities, such reported cases likely comprise only a minority of the total number of people affected. Therefore, exploring the full extent of the problem is often recommended by conducting thorough surveillance of the population at risk of foodborne illness via an active search for additional cases using ACD. The appropriate methods for detecting any other unreported cases will usually depend on the overall distribution and scale of such an outbreak, which varies according to the circumstances of a particular outbreak. Many FBD

outbreaks will usually involve groups of people who can easily be identifiable (for example, all persons attending the same wedding party), making case-finding via ACD simple.

Each of these cases of food poisoning was initially identified during the first phase of case detection via PCD, conducted by Infectious Disease Control Unit (IDCU) in Pahang via its routine surveillance activities. The second phase of case detection, known as ACD, was undertaken by authorised healthcare personnel from the Food Safety Quality (FSQ) Unit when they conducted interviews to ensure that all cases of food poisoning had been duly identified.

Results of Microbiological Analysis

All the laboratory test methods were performed according to the established national methods of food hygiene analysis (WHO, 2008). Results on microbiology identification of reported food poisoning incidents in Pahang from 2013-2018 were obtained from the Food Microbiology Laboratory at FSQ in Mentakab, Pahang. The identification and isolation of *Vibrio parahaemolyticus*, *Salmonella* spp., *E. coli*, *Staphylococcus aureus*, and *Bacillus cereus* within these contaminated food samples were further analysed in conjunction with these food poisoning incidents to determine their association with such food poisoning outbreaks. This association can then be useful for assessing the roles of these food pathogens as potential causative factors for these outbreaks.

Data Analysis

Specifically, the total incidence rates of all confirmed food poisoning cases from 2013 to 2018 were described, and cases within each of the 11 districts of Pahang were spatially mapped. In addition, descriptive analyses were performed to determine the sociodemographic and temporal characteristics of food poisoning cases (such as the locations and premises in which these cases occurred and the period within a year during which these cases occurred) within the state of Pahang. Finally, a list of contributory factors of all these food poisoning cases was identified, and each frequency (to be expressed in percentages) was tabulated. All these descriptive statistics were performed using Microsoft Excel (version 2016).

Ethical Clearance

This study was registered in National Medical Research Register (NMRR), Malaysia, and the NMRR ID for this study is NMRR-19-2979-51363. The Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia, has also given ethical approval for this study. Apart from making an adequate effort to ensure that this study collected no personal identifiers. The study would not jeopardise any individuals' rights and/or interests; the investigators were closely adhering to the institutional research guidelines (upon which this study approval was based) to maintain its scientific soundness and ethical conduct.

RESULTS AND DISCUSSION

Food Poisoning Incidents and Their Distribution

Present results showed that the number of reported cases fluctuated from 2013 until 2018, with an average of around 21 cases per year. No significant deterioration or improvement was seen over these years. However, a breakdown of the reported cases for each month of the year identified August as the month having the most such reported cases and May as the month having the fewest reported cases (Figure 1). It is interesting to note the high case in May of 2014 throughout the six years analysis.

Food poisoning occurred mostly during the third and fourth quarters of the year, with fewer reported cases occurring during the earlier part of the year (Table 1). It has probably arisen from the fact that the climatic conditions in Kuantan, the state capital of Pahang, are affected by the north-east monsoon wind, which initiates the monsoon season, which lasts for three months from November until January (Malaysian Meteorological Department [MetMalaysia], 2014), during which there is an unusually high level of rainfall. This heavy rainfall in Kuantan, which occurs during the monsoon season, tends to bring

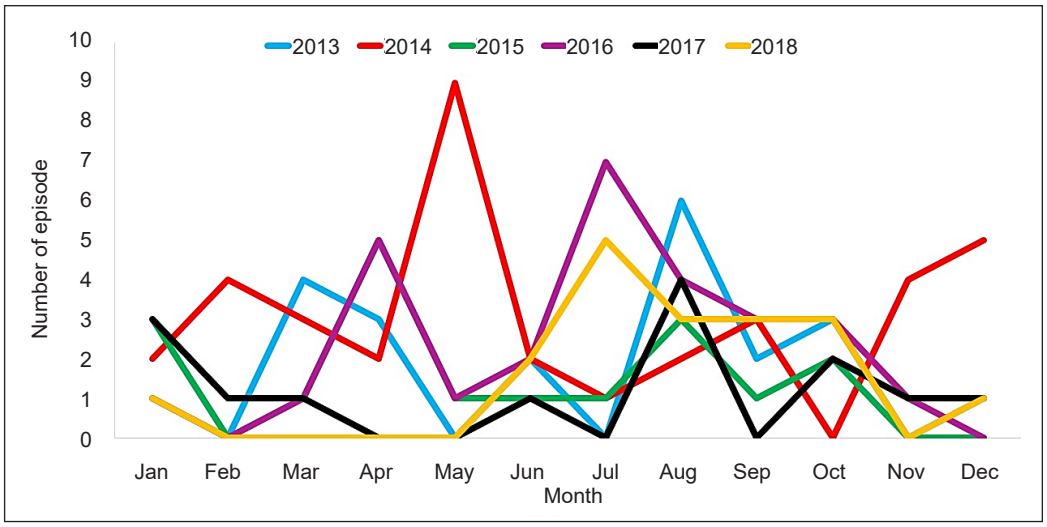

Figure 1. The trend of food poisoning episodes was reported every month in Pahang state from 2013 until 2018

Table 1
Food poisoning episodes that were reported in Pahang state from 2013 until 2018

Year / Quarter	2013	2014	2015	2016	2017	2018
1st	5	9	4	2	5	1
2nd	5	4	7	8	1	2
3rd	8	6	5	14	4	11
4th	4	9	2	4	4	4

about flooding within the surrounding regions in Pahang; it could also increase the risk of both foodborne and waterborne diseases occurring in Pahang, including food poisoning.

Close examination of the geographical distribution of food poisoning (Figure 2) found that Kuantan, as the state capital had an exceptionally high number of food poisoning incidents with a total of 48 episodes over the past six years, while only one episode was reported in Kuala Lipis in 2016, with sporadic episodes in the other districts such as Pekan and Raub.

Alternatively, a district with a larger population like Temerloh had reported more episodes of food poisoning cases when compared to others, with a total of 13 episodes over the past six years. Likewise, Rompin had similar observations with a higher count of food poisoning cases than other districts with 22 episodes. In addition, the most common locations where food poisoning was reported to occur were public schools (Table 2), with the highest number of cases reported in the boarding school kitchen, followed by the school canteen, and only one reported in a local restaurant.

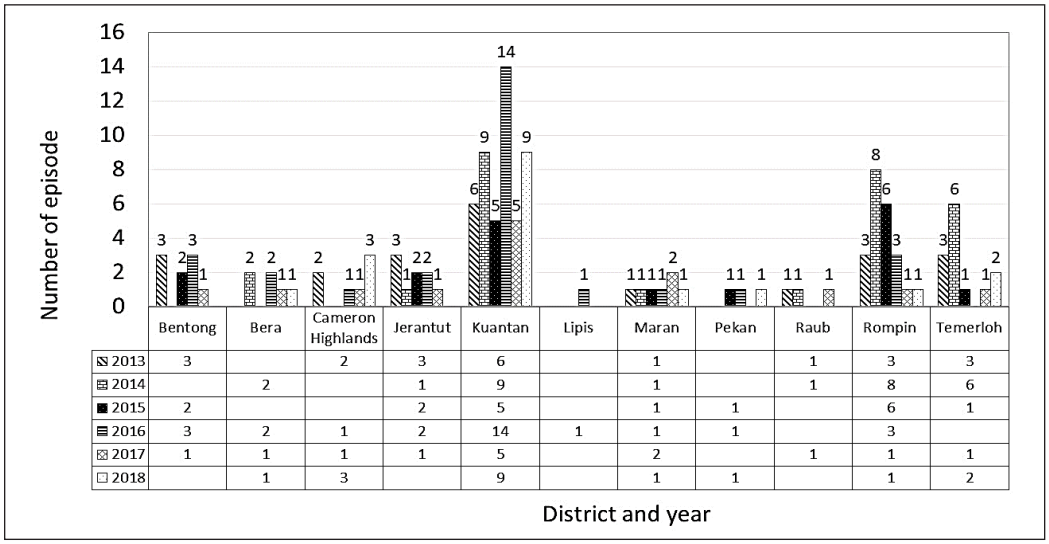

Figure 2. Food poisoning episodes that were reported in Pahang state districts from 2013 until 2018

Table 2
Food poisoning incidents that were reported in Pahang state, according to the facility, from 2013 until 2018

Facility \ Year	2013	2014	2015	2016	2017	2018
Public schools	15	17	10	18	6	15
Facility for National Service Training Program	-	-	-	-	1	-
Facility for matriculation program and higher learning	1	2	-	1	1	1
Others	6	9	7	9	6	3
Total	22	28	17	28	14	19

Previous studies had reported that whenever food items are cooked before consumption, leftover from a previous meal, or left overnight; then additional food processing steps include prolonged storage of food and, if necessary, reheating before serving (Griffith & Worsfold, 1994); can potentially lead to outbreaks of bacterial FBD if appropriate food safety measures have not been taken (Bryan, 1988; Knabel, 1995; Pelczar Jr et al., 1993). Hence, this rationalises the occurrence of food poisoning outbreaks in the canteens of boarding schools. Potential factors are associated with a heightened risk of food poisoning outbreaks within a large-scale food preparation operation.

The following section provides a detailed review of the entire food preparation process, which starts from the procurement of raw materials and fresh produce and subsequent storage of these materials for cooking, which will then be followed by a careful delineation of factors that can potentially affect the level of food hygiene during the handling of raw materials and cooked food during the daily routines of cooking, transporting, and storing the cooked food.

Procurement and Storage of Raw Materials and Fresh Produce

For large-scale production of cooked foods, it is first necessary to purchase the raw materials for the foods in bulk. Once these raw materials for the bulk foods have been transported to the kitchens, it is necessary to store them at optimal temperature and humidity conditions to prevent premature spoilage by ensuring that each type of food is kept at its ideal temperature and the proper light and moisture level. Raw meat and poultry are mostly supplied in a chilled or frozen state (to preserve their shelf-life) and are often regarded as high-risk food. If it becomes necessary to maintain such low temperatures throughout its handling time, then its transport time should be minimised, and an insulated cool bag should be used to carry and transport these food items around; to limit the growth of food spoilage bacteria (Griffith & Worsfold, 1994; Jay et al., 1999). Therefore, some of the food items being transported are likely exposed to the growth of pathogenic spoilage bacteria. At the same time, en route to the kitchen, which could potentially result in an incident of food poisoning. Therefore, as a precaution, all food handlers should take adequate measures to store and handle these food items hygienically upon transporting them to the kitchen to minimise the risk of contamination and prevent microbial overgrowth (Gorman et al., 2002; Griffith & Worsfold, 1994). Many studies conducted in the United Kingdom and Australia had indicated that consumers often failed to adhere to bacterial FBD prevention guidelines for ensuring food safety, such as storing high-risk food products at or below 4^{o}C to prevent microbial overgrowth, storing raw and ready-to-eat food products separately to minimise the risk of cross-contamination, and following the correct food safety procedures when thawing frozen food items to keep out of the "danger zone"

of temperatures to prevent food pathogens from thriving (Jay et al., 1999). If any of these safe food-handling practices are not implemented, then it becomes possible for an outbreak of food poisoning to occur.

General and Personal Hygiene During Food Preparation

The WHO data indicate that most FBD episodes that occur throughout the world are attributed to a small number of factors related to food handling; and two of the common errors include allowing people with poor personal hygiene to handle the food and adopting unhygienic practices that can potentially result in cross-contamination (WHO, 2008). One common way for causing cross-contamination results in food poisoning within the food industry is poor hygiene levels when handling and processing food by the food service providers, especially the food handlers (Sani & Siow, 2014). Therefore, prevention of food poisoning always refers to the adherence to safe practices during the daily routines in preparing, handling, and storing food. Hence, as a scientific discipline, the scope of food safety shall draw its perspectives from a wide range of academic fields, including food chemistry, food microbiology, and food engineering. Furthermore, from farm to factory to plate, it is likely for these food products to be exposed to many health hazards at every step during their journey through the whole supply chain. Thus, it is advisable to carefully implement safe food handling procedures at every stage of the food production supply chain to curb the spread of foodborne pathogens and prevent harm to consumers.

Most epidemiological data indicate that cross-contamination during food preparation and transport can result in FBDs (Forsythe & Hayes, 1998). Therefore, the manipulators (WHO, 2022) and the food will have to be carefully monitored at every step of handling, preparing, and transporting food (Gilling et al., 2001) to ensure that food remains microbiologically safe. There are several sources of microbial contamination, which are commonly identified as (a) unhygienic practices in handling food; (b) raw materials for cooking food and raw meat; (c) failure to implement adequate cleaning of the machines and equipment used to cut the food, and of those contact surfaces used to prepare the food (including the food handlers' and food manipulators' clothes and hands); and (d) airborne contamination (de Sousa, 2008). It underscores the high importance of a satisfactory level of hygiene to be observed by food manipulators during food preparation.

Handling of Cooked and Leftover Food

WHO data also indicate that two of the common errors include (a) preparation of food several hours before consumption and storing of cooked food at temperatures that favour overgrowth of food microbes and/or formation of their toxins; and (b) failure to allow sufficient cooking or reheating of food. Since food safety is a systematic approach to food hygiene that shoulders a responsibility that addresses every aspect of the global food industry, it is thus an important aspect

of public health linked to agriculture and other food production sectors (Schlundt, 2002). Several factors mainly lead to an outbreak of food poisoning, including (a) inadequate food manipulation to maintain high hygiene levels and prevent cross-contamination; (b) inappropriate holding temperatures (failure to refrigerate food at a sufficiently low temperature); (c) inadequate cooking and/or reheating; (d) contaminated equipment for food preparation and storage (the omission of cleaning and disinfecting kitchen or processing plant equipment); and (e) poor personal hygiene. Other factors that may contribute to the occurrence of food-borne illness include: (f) preparing food a day or more in advance before serving along with unsafe procedures for holding and reheating; (g) cross-contamination (from raw to cooked products); and (h) inadvertently introducing contaminated ingredients to cooked food. After foods have been contaminated, the main factor which greatly contributes to the occurrence of food poisoning outbreaks is allowing them to remain at a temperature that promotes the growth of the potentially hazardous microorganisms or their toxin production in the food (de Sousa, 2008). It highlights the significance of properly handling cooked and leftover food on the kitchen premises.

It is noteworthy to mention several important considerations from the perspective of a large-scale operation for food preparation. Apart from the usual hygienic practices that all food handlers must adopt, they should also be aware of the importance of safe storage of cooked food and leftover food, especially concerning its temperature and humidity conditions. Food handlers must remember that improper food storage may pose a health hazard to consumers. Furthermore, they will also need to understand the effect of optimal temperature for cooking and storing foods which can potentially influence bacterial growth. It underscores the importance of providing adequate training for food handlers in all aspects of safe handling of cooked and leftover food, such as storage and reheating. Previous studies reported that training for food handlers might improve their knowledge, practice, and hygiene awareness in food safety (Thompson et al., 2005) because it was reported that improving food safety knowledge through training positively affected food handling practices (Medeiros et al., 2011).

However, such training may not always bring about a positive change in food handling behaviour (Clayton et al., 2002; Seaman & Eves, 2006). One plausible explanation for this is provided by Clayton et al. (2002), who pointed out that food handlers may understand the need to conduct certain practices, but it would be too difficult, if not impossible, to implement them without having adequate resources. A recent study conducted in the food courts at Putrajaya revealed many issues about the food safety knowledge, attitude, and practices (KAP) of food handlers and the level of cleanliness of food courts in Putrajaya. This study's results showed that a mean score of 84.1% and 91.4% for food handlers' knowledge and attitudes levels

can be regarded as "high". However, a mean score of 79.5% for both knowledge and attitude would not necessarily be able to turn into safe practices (Siau et al., 2015).

This finding concurred with those from a previous study conducted in a school food service, and it was established that although the food safety knowledge was high, the safe food handling procedures were still not being implemented during food preparation (Henroid Jr & Sneed, 2004). It is apparent that although food handlers have an adequate mastery of knowledge on food safety, the lack of physical facilities and/or resources might be an obstacle to implementing proper food safety practices. Thus, when implementing safe food handling procedures, there is a need to ensure appropriate physical facilities and adequate resources are available to these food handlers.

Identified Food Pathogens and Their Associated Vehicles

Foodborne microbes cause a major problem of adversely affecting food safety and cause human infections after consuming animal products contaminated with microorganisms or their toxins (Heredia & Garcia, 2018). Previous studies had reported that Gram-negative bacteria accounted for approximately 69% of the cases of bacterial food-borne disease (Kebede et al., 2014). Although a total of 31 pathogens were identified as causing FBDs (Zhao et al., 2014), the more common causes of food-borne diseases and their related deaths in the world are mainly reported to be attributed to bacterial pathogens including *S. aureus*, *Salmonella* spp., *Campylobacter* spp., *L. monocytogenes*, and *E. coli* (Assefa & Bihon, 2018; Bantawa et al., 2018; Elmonir et al., 2018; Hemalata & Virupakshaiah, 2016; Zhao et al., 2014).

Many previous studies suggested that emphasising preventative food safety could contribute significantly to reducing the occurrence of food poisoning outbreaks (Osimani et al., 2011). Such an emphasis on the adoption of appropriate hygienic practices was based on published evidence of food safety, and these are documented in various aspects of food hygiene, such as on ready-to-eat meals, as well as on knowledge and attitudes on the implementation of effective hygiene intervention strategies (Buccheri et al., 2010). Thus, adequate measures should consistently be taken throughout food preparation to adopt a satisfactory level of hygienic practices to implement preventative food safety.

Among the suspected causative agents of food poisoning for the year 2018, *B. cereus* was one of the most frequently identified microbial pathogens, which had been reported to be linked to 8 cases of food poisoning (Table 3).

Table 3
Frequency for isolation of the identified microbial pathogen from food poisoning incidents in Pahang in 2018

Food pathogen	Frequency of cases of food poisoning (n) (%)
Bacillus cereus	8 (88.8%)
Staphylococcus aureus	6 (66.6%)
Salmonella spp.	5 (55.5%)
Vibrio parahaemolyticus	2 (22.2%)

It is already well-known that infection by *B. cereus* during food poisoning can cause either diarrhoea syndrome or emesis syndrome because it produces two types of toxins: emetic (vomiting) and diarrhoeal, which can result in two types of illnesses, which probably explains its particularly high incidence of food poisoning cases. In addition, another six cases of *S. aureus* infection were reported, followed by five cases of *Salmonella* spp. infection and two cases of food poisoning caused by *V. parahaemolyticus* were detected in 2018. Present findings agree with Su et al. (2005). They further reiterated that the trends of indigenous FBD within the Asian area had shown that the most important foodborne pathogens are *V. parahaemolyticus*, *S. aureus*, and *Salmonella* spp. based on their association with a high disease burden (Su et al., 2005).

Although *B. cereus* is not usually regarded as a common food pathogen that is isolated from food poisoning incidents, however, *B. cereus* food poisoning often results from its thermoduric endospore, which enables it to survive in cold temperatures and doubles its population within a short period of times depending on the food product (Kotiranta et al., 2000). Therefore, it is necessary to either avoid consuming any contaminated food, which contains large numbers of bacterial cells and/or spores of *B. cereus* or avoids consuming food contaminated with the pre-formed toxin of *B. cereus* by implementing proper procedures for food handling/storage and cooling of cooked food (Schneider et al., 2004). Furthermore, for both the diarrhoeal and emetic types of *B. cereus* food poisoning, the food involved has usually been pre-treated by heat; hence, the heat-resistant spores or thermoduric endospores are the sources of food poisoning (Granum & Lund, 1997). Therefore, it again highlights the high significance of properly handling all cooked and leftover food.

Based on the perspectives of the operation for large-scale food production within premises such as boarding school kitchens or school canteens, the food can be served by these establishments to be contaminated with foodborne pathogens, which are the leading cause of acute diarrhoea, an initial presenting symptom of food poisoning. In a large-scale food preparation premise such as a boarding school kitchen or school canteen, the pathogen—food category pairs identified to be responsible for most single-pathogen food poisoning outbreaks will depend on the type of food being cooked and served. The main reason for this dependency is the eating habit. In Malaysia, the staple food for a typical daily diet will mostly consist of rice; hence this staple food which typically consists of rice and bean products is often identified as the main vehicle of *B. cereus* (Liu et al., 2017). Hence, it is likely that the contaminated rice serves as a vehicle for the transmission of *B. cereus* food poisoning in Malaysia. It also probably explains why *B. cereus* was one of the most identified microbial pathogens among all the suspected causative agents of food poisoning within Pahang, Malaysia, for the year 2018.

Other Contributory Factors to Food Poisoning Outbreaks

In Malaysia, the Malaysia Standard 1514:2001 is coded as the standard for users to define all the essential principles of food hygiene that apply to the whole supply chain, including the entire processing step, which begins from primary production to completion, where the finished product will be served to the final consumer (Talib & Ali, 2009). Since it will also guide different sectors of the food chain processes or commodities to amplify and further tailor each of these hygiene requirements to these sectors, therefore the main purpose of this standard is to serve as a benchmark for all the food handlers to adhere to it for ensuring that food is safe for human consumption (Talib & Ali, 2009).

According to Talib and Ali (2009), some of the hygiene principles covered include (a) environmental hygiene of primary production; (b) design of establishment and facilities; (c) control of operations; (d) maintenance and cleaning of the establishment (including pest control); (e) personal hygiene; (f) transportation; (g) labelling and lot identification of product information; and (h) training and supervision. Therefore, any breaches in the eight hygiene principles (as listed above) are likely to contribute to a food poisoning outbreak. Table 4 illustrates all the contributing factors to food poisoning outbreaks in Pahang from 2013 until 2018.

It shows that a lack of pest control is a major contributing factor with a high frequency of 18.64%, followed by cross-contamination and inadequate food manufacturing process control as a secondary contributing factor, each with a frequency of 13.56%. As alluded to earlier, the total number of food poisoning incidents that occurred in the boarding schools was much higher than that in non-boarding schools, and since the communal kitchens in these boarding schools were designed to store large amounts of raw materials for cooking (since the cooks were cooking in large quantities in these communal kitchens

Table 4
Eleven food poisoning-causing factors

Contributing factor	Percentage (%)
Lack of pest infestation control	18.64
Cross-contamination during food preparation	13.56
Inadequate control of the food manufacturing process	13.56
Poor maintenance of equipment or building	11.3
Contamination by food handlers	10.17
Lack of cleaning program	8.47
Lack of hand washing facility	7.91
Various uncontrolled factors	7.91
Lack of proper storage	6.78
Contamination with chemicals	1.13
Lack of vehicle suitable for foods	0.56

when they prepare meals for a large number of boarding school residents at any one time); there is a tendency for the problem of pest infestation to crop up.

It is a common problem in the storage of raw materials for food because contamination of such raw materials during storage can often occur as a result of pest infestation and/or failure to keep them in wrapped or closed containers (Wisner & Adams, 2002), which explains why the practice of effective pest control for the storage of these raw materials is regarded with high importance. In this regard, the Food Hygiene Regulations 2009 contains several provisions which can be highly relevant for ensuring a high level of hygiene and sanitation in the food industry, promoting hygienic practices among food handlers, and maintaining a satisfactory standard of cleanliness within the food premise (Ismail, 2011), which included any premises used for or concerned with preparing and handling all types of foods; including their relabelling, reprocessing or reconditioning (Food Act 1983, 2012). These provisions can be very useful for instituting adequate pest control within institutions where large-scale food preparation is undertaken.

Likewise, many studies had already pinpointed the fact that many types of food pathogens might be transferred to the food items by cross-contamination through hands, surfaces, utensils, and other equipment that was not adequately cleaned and disinfected during the process for the preparation of different types of food, or between the different steps during the preparation of the same type of food (Roberts, 1990; Scott & Bloomfield, 1990); which would partially rationalise the finding that cross-contamination was identified to be a secondary contributing factor towards such food poisoning outbreaks in Pahang. Such a finding concurred with what was reported by many studies that cross-contamination can easily occur during processing, preparation, delivery, and service steps (Carrasco et al., 2012). In addition, previous studies reported that food handlers who failed to implement appropriate food handling practices often contributed to outbreaks of FBD in the food industry (Bryan, 1988).

In addition, the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention (ECDC) stated that food handlers are one of the two common contributory factors of FBDs, including food poisoning (European Food Safety Authority [EFSA] & European Centre for Disease Prevention and Control [ECDC], 2015). EFSA and ECDC (2015)'s scientific report shows that food handlers infected with food-borne disease pathogens had contributed to 7.3% of reported food-borne disease outbreaks. Furthermore, the report also stated that food handlers mishandling food (whether raw or cooked) concerning their storage temperatures had also contributed to 3.9% of reported FBD outbreaks. Cross-contamination from inappropriate and unhygienic food handler practices contributed to 3.2% of reported FBD outbreaks in 2014 (EFSA & ECDC, 2015).

In the third place, contributing factor to food poisoning outbreaks in Pahang was inadequate food manufacturing process control. The Malaysian Food Safety and Quality Division Annual Report 2012 states that "the measures for food safety assurance were developed, implemented, and monitored to further improve food safety by reducing food contamination and the occurrence of food poisoning". As such, many training programs and teaching activities related to the institution of food safety assurance were launched to train and impart the importance of food safety to all these employees working on food premises (Food Safety and Quality Division [FSQD], 2012). Examples of steps taken by food handlers to maintain food safety include the need to maintain good personal hygiene, to be aware of crucial aspects of food safety which link to temperature values with cooking temperature necessary for the control of microbiological hazards to limit the microbial overgrowth, and to take proactive steps to minimise any possible adverse effect of temperature during cooking of foods which can promote bacterial growth in food. All these are directly related to the adequacy of instituting control measures during food preparation. In a large-scale commercial food production where food is prepared in large quantities, especially within an institution such as the boarding school kitchen or school canteen; the food handlers might often be the agent or vector in spreading viruses if they fail to observe or adhere to any of the appropriate food safety and good food handling practices (Seaman & Eves, 2010).

Moreover, it can often happen during the short period to prepare dishes from raw foods; because food handlers might have forgotten to follow good food handling practices when they leave dish uncovered for an excessively long time or fail to clean their hands between handling raw and cooked foods (Toh & Birchenough, 2000). Indeed, previous research findings had also reported that a lack of awareness of the importance of temperature control was the main critical control point in the food preparation process, which could often hinder the successful implementation of an effective food safety program (Siau et al., 2015).

CONCLUSION

The findings from this study have provided an overview of the total number of food poisoning incidents that had occurred within Pahang over this 6-year period, which had greatly escalated during the period from September to November each year; and also identified *B. cereus* and *S. aureus* to be the two most common agents who were implicated in food poisoning cases within the Pahang state. These findings shall enable us to pay attention to particular months during the year when there will be a higher probability of food poisoning incidents. In addition, inadequate measures were taken for pest control and the heightened risk of cross-contamination during food handling (which includes transport and delivery of both raw materials and cooked food), as well as inadequate control of the food manufacturing process (including

adequate temperature control and proper storage conditions) were identified as critical factors with a major contribution towards such food poisoning outbreaks in Pahang. Finally, better quality research shall also be conducted in the future by using a more rigorous survey methodology to elicit a more thorough understanding of the potential causes of these food poisoning outbreaks and then to heighten the overall level of awareness by alerting the relevant authorities to focus their efforts in mobilising greater quantity of resources for mitigating this problem.

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Potential of Plant Growth Regulators to Enhance Arsenic Phytostabilization by *Pennisetum purpureum* cv. Mott

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ABSTRACT

The limited translocation of arsenic from contaminated soil to plant biomass is one way to decrease human exposure to arsenic (As). Plant growth regulators (PGR), including salicylic acid, indole butyric acid, and calcium, have been reported to alleviate toxicity and decrease the accumulation of heavy metals in many plants. Thus, this study has investigated the effect of plant growth regulators, including salicylic acid, salicylic acid + calcium chloride, indole butyric acid, and indole butyric acid + calcium chloride, to stimulate the growth and phytostabilization of *Pennisetum purpureum* cv. Mott grew in arsenic-spiked soil. The results showed shoot growth, root growth, and total chlorophyll content of *P. purpureum* cv. Mott grown in non-spiked soil were not significantly different from those grown in arsenic-spiked soil. Only the root-to-shoot ratio of plants grown under arsenic-spiked soil (0.28) was higher than that of non-spiked soil (0.19). Exogenous plant growth regulator application of each formula did not stimulate the growth of plants grown under both soil conditions. The most suitable plant growth regulator was indole butyric acid + calcium chloride, as the highest arsenic accumulation in plant roots was detected (47.38 mg/kg). It corresponds with the arsenic bioaccumulation factor, translocation factor, and efficiency, which were 4.52, 0.06, and 9.77% when using exogenously indole butyric acid + calcium chloride. Meanwhile, arsenic's translocation factor and efficiency were low when using the other formulae of plant growth regulators. Thus, 0.001 mM indole butyric acid + 20 mM calcium chloride may be used for the cultivation of *P.*

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purpureum cv. Mott as a forage crop in areas with low levels of arsenic contamination because it could limit the amount of arsenic entering the food chain.

Keywords: Arsenic, Napier grass, phytoremediation, plant growth regulator

INTRODUCTION

Arsenic is a hazardous metalloid naturally found in the Earth's crust with an average concentration of 1–2 mg/kg (Sanyal et al., 2020). Increases in the concentration of arsenic in the soil and groundwater usually come from geological reactions and anthropogenic activity including mining, smelting, or other industrial activities (Mateo et al., 2019; Mitra et al., 2017). Arsenic can be a contaminant in agricultural areas when using inorganic arsenic as a pesticide or defoliant (Wani et al., 2017). Millions of people worldwide are in danger from arsenic toxicity, and exposure to arsenic is a public health concern in several countries in Asia, including China, India, Thailand, and Vietnam (Mitra et al., 2017; Sanyal et al., 2020). In Thailand, the average concentration of arsenic in agricultural soil ranges from less than 0.005 to 64 mg/kg, and the mean value was 5.8 ± 6.5 mg/kg (Sukreeyapongse et al., 2009). The maximum concentration of arsenic in soil and agricultural soil specified by the Office of the National Environment Board of Thailand was 3.9 mg/kg (Weerasiri et al., 2014). The most prevalent inorganic forms of arsenic in the environment were arsenite ($H_2AsO_4^-$; As^{III+}) and arsenate (H_3AsO_3; As^{V+}); however, arsenic in the organic form was seldom detected in the environment (Sanyal et al., 2020). The toxicity of arsenic depends on the prevalent form in the environment. For example, arsenate is less toxic, while arsenite is more toxic and movable than arsenate because it has a higher ability to bind with functional biomolecules, such as sulfhydryl groups and cysteinyl residues (Berg & Borges, 2020; Mateo et al., 2019). Contamination of arsenic in soil is an environmental concern because arsenic is classified as a Group I human carcinogen, and it has several toxic effects on humans, including hypopigmentation, pigmentation, keratosis, and skin cancers (Sanyal et al., 2020). The route for human arsenic exposure is mainly from drinking contaminated water, eating contaminated food, direct skin contact with contaminated soil, and inhaling contaminated dust (Loukola-Ruskeeniemi et al., 2022). Biomagnification of arsenic along the food chain is a major concern of arsenic exposure in humans because arsenic has been reported in food crops in Poaceae plants, such as rice, maize, sorghum, and wheat (Upadhyay et al., 2019).

Based on the toxic health effects and biomagnification capacity along the food chain of arsenic, arsenic remediation from contaminated sites to decrease human contact with arsenic is a priority task. Among several arsenic decontamination methods, phytoremediation is feasible, environmentally friendly, and cost-effective in removing arsenic from contaminated sites (Mateo et al., 2019). Several phytoremediation mechanisms can be used

for arsenic decontamination, including phytoextraction and phytostabilization. Several terrestrial plant species have been used to remove arsenic from contaminated soil via phytoextraction, including *Bambusa bambos*, *Pennisetum purpureum*, *Vetiveria zizanioides* (Sampanpanish & Suwattiga, 2017), *Zea mays* (Mehmood et al., 2021), *Salix purpurea* 'Fish Creek', *Festuca arudinacea*, *Medicago sativa*, and *Brassica juncea* (Yanitch et al., 2020). The phytostabilization of arsenic has been reported less than phytoextraction, but it is still interesting. In addition, some plants have been reported to phytostabilize arsenic, including dwarf Napier grass (Boonmeerati & Sampanpanish, 2021; Kowitwiwat & Sampanpanish, 2020) and the halophyte *Acanthus ilicifolius* (Sarath et al., 2022). These plants were reported for use in mine tailing remediation. Even though the success of phytostabilization of arsenic differed from site to site, the accomplishment of the process depends on several factors in the environment, such as water, nutrients, oxygen soil structure, pH, and presence of other toxins (Hauptvogl et al., 2020). For example, the arsenic phytostabilization by *Phragmites australis* and *Arundo donax* were increased when compost was an amendment in planted soil (Castaldi et al., 2018).

Phytostabilization promises to be ecosystem friendly due to promoting the soil development process, microbial diversity, and self-reliance for environmental restoration as a long-term goal (Zine et al., 2020). The main hindrance to arsenic phytoremediation is plant growth inhibition due to the high concentration of arsenic, which also retards the phytoremediation efficiency (Mateo et al., 2019). Plant growth under arsenic contamination usually causes oxidative stress (A. P. Singh et al., 2017) and reduced photosynthetic pigments in plants (Kumari & Pandey-Rai, 2018). Decreasing metal toxicity to plants is important to improve the phytostabilization process, especially for mine waste remediation (Zine et al., 2020). In the case of arsenic contamination, the application of exogenous plant growth regulators, including salicylic acid, auxin, and calcium, is one way to improve plant growth by alleviating arsenic toxicity in plants grown under arsenic stress (He et al., 2021; Maghsoudi et al., 2020; R. Singh et al., 2018, 2020). The plant growth regulators in this study could protect plants from arsenic stress with several mechanisms. Salicylic acid could alter the plant's physiological and metabolic processes and alleviate the environmental stress on plants (Arfi et al., 2020). The salicylic acid application can enhance antioxidant enzymes and decrease arsenic accumulation in the plant (Maghsoudi et al., 2020). Exogenous auxin also mitigates arsenic toxicity in the plant by decreasing the arsenic translocation from the root to the shoot and promotes plant growth by increasing plant biomass, relative root length, relative root fresh weight, relative root length (He et al., 2021), and other negative impacts from arsenic toxicity (Piacentini et al., 2020). Meanwhile, calcium can act as a secondary

messenger and control signal transduction in plants grown under stress and non-stress conditions. Modulating key antioxidant enzymes and stabilized membranes by calcium plays an important role in protecting plants grown under environmental stress (R. Singh et al., 2018, 2020). Exogenous calcium application also reduced arsenic uptake and increased antioxidant enzymes in a plant under arsenic stress (Rahman et al., 2015). For the reasons described above, the objective of this study was to investigate the roles of salicylic acid and indolebutyric acid application alone or in combination with calcium to stimulate the growth and phytostabilization of arsenic by *Pennisetum purpureum* cv. Mott in a pot experiment. *Pennisetum purpureum* cv. Mott (dwarf Napier grass) was used as a model plant to stabilize arsenic from the soil in this study because this plant species has been reported to remediate arsenic via phytostabilization (Kowitwiwat & Sampanpanish, 2020). Moreover, this plant can grow in several soil types and weather conditions, it is resistant to many pests, and its biomass is used for animal feed and as a substrate for bioethanol production (Ishii et al., 2015). These plant growth regulators have been reported to decrease arsenic accumulation in plants in the Poaceae family, such as rice and wheat (He et al., 2021; Maghsoudi et al., 2020). Suppose these three plant growth regulators could increase arsenic phytostabilization of *P. purpureum* cv. Mott, the shoot biomass of the grass will be safe to be used for feed for livestock or bioenergy production in the future.

MATERIALS AND METHODS

Preparation of Arsenic-Spiked Soil

The soil used in this study was collected from an agricultural area in Muang Pluai Sub-District, Srisomdej District, Roi-Et Province, Thailand. The soil was air dry for two weeks, sieved with a 2 mm sieve, and sent for analysis of its physical and chemical characteristics at the Central Laboratory Thailand, Ltd. (Khon Kaen branch). The texture of the soil was loamy sand with 81.9% sand, 13.16% silt, 4.95% clay, pH 5.71, cation exchange capacity was 3.42 mg/100g, and the background level of arsenic in the soil was 0.809 mg/kg. Then, 1 kg of soil was weighed and used in each pot for the experiment. One kg of soil in each pot was spiked with 1,000 ± 0.005 g/l of standard arsenic solution (ChemSupply, Australia) and left to dry at room temperature for five days. The soil was mixed thoroughly several times to ensure it was homogenous before use. Soil samples were collected to determine the arsenic concentration in the soil after spiking, and the final concentration of the arsenic in the soil was 12.92 ± 0.54 mg/kg. Soil without arsenic spiking was used in the control pots. The soil pH after being spiked with the standard arsenic solution was approximately 3.9, and the pH of the control soil without arsenic addition was adjusted to 3.9 with a 2% (w/w) nitric acid solution (ANaPURE, New Zealand). Soil moisture content after soaking the soil with water was 11.67%.

Pot Experiment

A farmer kindly provided *P. purpureum* cv. Mott in Phon-Ngam Sub-District, Kamalasai District, Thailand. Cuttings of *P. purpureum* cv. Mott were cut into similar sized (approximately 8-10 cm per piece) and immersed in 1 mM salicylic acid (Sigma-Aldrich, USA), 0.001 mM indolebutyric acid (Fluka, China), 1 mM salicylic acid (Sigma-Aldrich, USA) + 20 mM calcium chloride (Ajax Finechem Pty Ltd., New Zealand), 0.001 mM indolebutyric acid (Fluka, China) + 20 mM calcium chloride (Ajax Finechem Pty Ltd., New Zealand) or water for three days. Then, similar-sized cuttings were planted into arsenic-spiked soil or non-spiked soil according to the treatments described below. One cutting was planted in each experimental pot. The experiment was performed with a completely randomized design (CRD) with 2 x 5 factors. The first factor was two levels of arsenic contamination (non-spiked soil and arsenic-spiked soils), and the second factor was five levels of plant growth regulator application (no plant growth regulator, salicylic acid, salicylic acid + calcium chloride, indole butyric acid, and indole butyric acid + calcium chloride). There were 10 treatments, and each treatment was performed in eight replicates, as described below:

1. Non-spiked soil + plant
2. Non-spiked soil + plant + salicylic acid
3. Non-spiked soil + plant + indole butyric acid
4. Non-spiked soil + plant + salicylic acid + calcium chloride
5. Non-spiked soil + plant + indole butyric acid + calcium chloride
6. Arsenic-spiked soil + plant
7. Arsenic-spiked soil + plant + salicylic acid
8. Arsenic-spiked soil + plant + indole butyric acid
9. Arsenic-spiked soil + plant + salicylic acid + calcium chloride
10. Arsenic-spiked soil + plant + indole butyric acid + calcium chloride
11. Arsenic-spiked soil

After transferring the cuttings into the experimental pots, 240 ml of 1/16 concentration Hoagland modified basal salt mixture solution (PhytoTech Labs, USA) was poured into each pot at the beginning of the experiment. Water was irrigated to the experimental pots every day for seven days after transplantation, and then irrigation was changed to every three days until the end of the experiment. Then, 10 ml of each formula of the plant growth regulators (1 mM salicylic acid, 0.001 mM indolebutyric acid, 1 mM salicylic acid + 20 mM calcium chloride, 0.001 mM indolebutyric acid + 20 mM calcium chloride) was poured into the soil planted with *P. purpureum* cv. Mott again on days 15 and 25 after transplanting. The experiment was terminated 41 days after transplanting.

Plant Growth Parameters, Arsenic Remaining in Soil, and Plant Biomass

At the end of the experiment, the plant growth parameters were determined, including leaf number, stem number, shoot length, root length, shoot fresh weight,

shoot dry weight, root fresh weight, root dry weight, and chlorophyll contents in the leaves. Total chlorophyll, chlorophyll *a*, and chlorophyll *b* contents were determined according to the methods described in Huang et al. (2004). Specific root length and root-to-shoot ratio were calculated from root length/root dry weight and root dry weight/ shoot dry weight according to the formulae described in Calvelo Pereira et al. (2010) and Xu et al. (2018), respectively. Soil and plant biomass were sent for analysis at the Central Laboratory Thailand, Ltd. (Khon Kaen branch) by the in-house method based on EPA3052 using inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent Model 7500, Japan). Then, the bioconcentration factor (BCF) and translocation factor (TF) of arsenic were calculated by the formulae described in Hammami et al. (2016). The bioconcentration factor was calculated from the total arsenic concentration in the harvested plant/total arsenic concentration in planted soil, and the translocation factor was calculated from the total arsenic concentration in the shoot/ total concentration in the root (Hammami et al., 2016). Moreover, the translocation efficiency (TE%) was calculated by the equation described in Hammami et al. (2016):

$$TE\% = [\text{arsenic concentration in shoot} / \text{arsenic concentration in whole plant}] \times 100$$

Statistical Analysis

A two-way analysis of variance (ANOVA) was used for variance analysis among treatments for the arsenic toxicity to plant growth. One-way ANOVA was used for variance analysis among treatments of the phytoremediation experiment. The least significant difference (LSD) method was used for pairwise comparisons of means. The data are shown as mean ± standard error (SE), and the statistical differences are shown as $P \leq 0.05$.

RESULTS AND DISCUSSION

Growth of Plants Under Arsenic Contamination

In general, the toxic effects of arsenic exposure on terrestrial plants are similar, such as causing oxidative stress from reactive oxygen species production (A. P. Singh et al., 2017), reducing total chlorophyll content (Kumari & Pandey-Rai, 2018), inducing chlorosis, reducing relative water content in the leaf (Rahman et al., 2015), alteration of auxin biosynthesis, and distribution in plant roots (Piacentini et al., 2020). In this study, these two factors, arsenic and plant growth regulator, affected plant growth differently, and there was no interaction between the factors. Arsenic decreases the shoot growth, chlorophyll *b*, total chlorophyll content, and root fresh weight of *P. purpureum* cv. Mott. While plant growth regulator application did not alter almost all plant growth of *P. purpureum* cv. Mott. Only applying salicylic acid and salicylic acid + calcium decreases the number of leaves per plant and chlorophyll content in leaves (Table 1). The growth of the plants in both arsenic-spiked and non-spiked soil was similar, and chlorosis was not detected by the naked eye (Figure 1).

Table 1
The main effect of arsenic and plant growth regulator on Napier growth traits (data shown as mean ± SE)

	Leave number / plant	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Chlorophyll *a* (mg/ml)	Chlorophyll *b* (mg/ml)	Total chlorophyll (mg/ml)
Arsenic (factor 1)										
Without As	24.2 ± 1.23a	36.2 ± 1.43a	49.9 ± 1.66a	6.3 ± 0.26a	34.8 ± 1.46a	14.5 ± 0.87a	1.2 ± 0.12a	28.2 ± 2.56a	16.7 ± 1.24a	44.9 ± 3.47a
With As	19.6 ± 1.23b	31.9 ± 1.43b	38.5 ± 1.66b	5.0 ± 0.26b	35.2 ± 1.46a	7.9 ± 0.87b	1.1 ± 0.12a	22.8 ± 2.44a	11.0 ± 1.18b	33.8 ± 3.31b
F-test	*	*	**	**	ns	*	ns	ns	**	*
Plant growth regulator (factor 2)										
No plant growth regulator	24.8 ± 1.94a	34.6 ± 2.26a	50.7 ± 2.62a	6.1 ± 0.41a	36.6 ± 2.31a	11.5 ± 1.38a	1.4 ± 0.18a	27.6 ± 3.86a	19.00 ± 1.86a	46.6 ± 5.23a
Salicylic acid	17.7 ± 1.94b	37.1 ± 2.26a	44.3 ± 2.62a	5.9 ± 0.41a	37.9 ± 2.31a	12.7 ± 1.38a	1.2 ± 0.18a	21.5 ± 4.32a	9.99 ± 2.08b	31.5 ± 5.84a
Salicylic acid + calcium chloride	19.9 ± 1.94ab	34.9 ± 2.26a	40.8 ± 2.62a	4.9 ± 0.41a	33.7 ± 2.31a	9.9 ± 1.38a	0.9 ± 0.18a	24.8 ± 3.86a	12.4 ± 1.86b	37.2 ± 5.23a
Indole butyric acid	25.2 ± 1.94a	29.5 ± 2.26a	43.0 ± 2.62a	6.0 ± 0.41a	32.9 ± 2.31a	11.6 ± 1.38a	1.2 ± 0.18a	25.2 ± 3.86a	14.6 ± 1.86ab	39.8 ± 5.23a
Indole butyric acid + calcium chloride	21.9 ± 1.94ab	34.2 ± 2.26a	42.2 ± 2.62a	5.4 ± 0.41a	34.0 ± 2.31a	10.4 ± 1.38a	1.0 ± 0.18a	28.4 ± 3.86a	13.2 ± 1.86b	41.6 ± 5.23a
F-test	*	ns	ns	ns	ns	ns	ns	ns	*	ns
F-test As x Plant growth regulator	ns	*	ns	ns	ns	ns	ns	ns	ns	ns

Note. Different lowercase letters show significant differences within each factor. Abbreviations: ns, *, ** denote non-significance ($P > 0.05$), statistical significance ($P < 0.05$), and high statistical significance ($P < 0.01$) of each factor, respectively

(a)

(b)

Figure 1. *Pennisetum purpureum* cv. Mott grew under: (a) non-spiked soil; and (b) arsenic-spiked soil in the absence of any plant growth regulators

The concentration of arsenic in this study seemed to be low, but it was in the range of the arsenic background levels in agricultural soil in Thailand (Weerasiri et al., 2014). *Pennisetum purpureum* cv. Mott has been reported to tolerate arsenic toxicity as it can grow and accumulate in both aboveground and belowground parts when growing in mine tailings with 68 ± 2.65 mg/kg of arsenic (Sampanpanish & Suwattiga et al., 2020). However, when compared between the same plant growth regulator application, shoot growth and root growth of the grass in arsenic-spiked soil were not significantly different from those grown in soil without arsenic. However, using salicylic acid, indole butyric acid, and indole butyric acid + calcium chloride decreased the shoot fresh weight and root fresh weight of *P. purpureum* cv. Mott grown in soil contaminated with arsenic compared to those without arsenic contamination (Tables 2 and 3). The shoot fresh weight and root fresh weight of *P. purpureum* cv. Mott was around 35.0–37.2 g and 6.9–8.7 g, respectively, when salicylic acid, indole butyric acid, or indole butyric acid + calcium chloride were applied in arsenic-contaminated soil. The addition of salicylic acid tended to decrease the stem number and leaf number of *P. purpureum* cv. Mott grew in arsenic-spiked soil. This effect was not detected in the leaf number of plants grown in non-spiked soil (Table 2). Shoot length, shoot fresh weight, and shoot dry weight of *P. purpureum* cv. Mott grown in soil with and without arsenic contamination were around 31.4–38.0 cm, 46.2–55.1 g, and 5.4–6.8 g, respectively (Table 2). Root length, root fresh weight, and root dry weight of *P. purpureum* cv. Mott grown in soil with and without arsenic contamination

were around 34.0–39.2 cm, 8.8–14.3 g, and 1.3–1.5 g, respectively (Table 3). Applying plant growth regulators (salicylic acid, salicylic acid + calcium chloride, indole butyric acid, or indole butyric acid + calcium chloride) did not stimulate the growth of the shoots and roots of *P. purpureum* cv. Mott grown under both soil conditions, even though auxin, salicylic acid, and calcium have previously been reported to stimulate the growth of other plants grown under arsenic contamination.

For example, exogenous application of indole-3-acetic acid and salicylic increased the relative dry weight, relative shoot length, and relative root elongation in rice grown in soil contaminated with arsenic at 28.7 ± 1.52 mg/kg (He et al. 2021). Exogenous application of calcium chloride also increased rice growth by restoration of chlorophyll damage, enhancing the dry weight and antioxidant system in rice seedlings after exposure to 0.5 and 1 mM of disodium arsenate (Na_2HAsO_4) (Rahman et al., 2015). However, the shoot and root fresh weight of plants grown in arsenic-contaminated soil tended to be lower than those grown in non-contaminated soil. It corresponds to the lower root/shoot ratio of *P. purpureum* cv. Mott grew in arsenic-spiked soil, especially for plants with salicylic acid and without plant growth

Table 2
Shoot growth of Pennisetum purpureum *cv. Mott in the presence of plant growth regulators under non-spiked and arsenic-spiked soil (data shown as mean ± SE)*

Treatment	Stems number/plant	Leave number/ plant	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)
Non-spiked soil					
No plant growth regulator	3.8 ± 0.30	26.1 ± 1.54aA	38.0 ± 2.08aA	55.1 ± 2.19aA	6.8 ± 0.46aA
Salicylic acid	2.9 ± 0.50	20.8 ± 3.12aA	39.8 ± 4.07aA	51.4 ± 4.05aA	6.6 ± 0.72aA
Salicylic acid + calcium chloride	2.8 ± 0.47	22.2 ± 2.79aA	32.1 ± 2.07aA	42.8 ± 3.68aA	5.1 ± 0.52aA
Indole butyric acid	4.0 ± 0.36	26.2 ± 1.63aA	32.8 ± 2.86aA	51.1 ± 4.10aA	7.0 ± 0.74aA
Indole butyric acid + calcium chloride	3.5 ± 0.68	25.4 ± 3.91aA	38.5 ± 4.29aA	49.0 ± 2.95aA	6.0 ± 0.52aA
As-spiked soil					
No plant growth regulator	3.2 ± 0.60	23.5 ± 3.52aA	31.4 ± 2.87aA	46.2 ± 4.77aA	5.4 ± 0.34aA
Salicylic acid	1.5 ± 0.18	14.6 ± 1.48bA	34.4 ± 2.35aA	37.2 ± 1.57aB	5.2 ± 0.42aA
Salicylic acid + calcium chloride	2.0 ± 0.36	17.5 ± 2.14abA	37.6 ± 2.98aA	38.8 ± 3.78aA	4.8 ± 0.64aA
Indole butyric acid	3.2 ± 0.35	24.1 ± 2.68aA	26.2 ± 3.37aA	35.0 ± 4.28aB	5.0 ± 0.56aB
Indole butyric acid + calcium chloride	2.6 ± 0.60	18.4 ± 2.50abA	29.8 ± 0.10aA	35.4 ± 3.16aB	4.8 ± 0.56aA

Note. Different lowercase letters show significant differences ($P < 0.05$) between plant growth regulators within the same As concentration; different capital letters show significant differences ($P < 0.05$) between with and without As at the same plant growth regulator

Table 3
Root growth of Pennisetum purpureum *cv. Mott in the presence of plant growth regulator under non-spiked and arsenic-spiked soil (data shown as mean ± SE)*

Treatment	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Root-to-shoot ratio	Specific root length (m/g)
Non-spiked soil					
No plant growth regulator	34.0 ± 2.43aA	14.3 ± 2.14aA	1.3 ± 0.19aA	0.19	0.27
Salicylic acid	39.7 ± 4.72aA	16.8 ± 2.23aA	1.3 ± 0.18aA	0.19	0.32
Salicylic acid + calcium chloride	31.5 ± 1.97aA	12.0 ± 2.28aA	0.9 ± 0.19aA	0.18	0.33
Indole butyric acid	34.0 ± 3.16aA	15.6 ± 2.33aA	1.5 ± 0.26aA	0.21	0.23
Indole butyric acid + calcium chloride	34.9 ± 2.16aA	14.0 ± 1.89aA	1.2 ± 0.20aA	0.20	0.28
As-spiked soil					
No plant growth regulator	39.2 ± 3.61aA	8.8 ± 1.48aA	1.5 ± 0.34aA	0.28	0.26
Salicylic acid	36.0 ± 3.22aA	8.7 ± 1.52aB	1.2 ± 0.33aA	0.23	0.30
Salicylic acid + calcium chloride	35.8 ± 3.61aA	7.9 ± 1.28aA	0.9 ± 0.16aA	0.18	0.42
Indole butyric acid	31.9 ± 3.50aA	7.5 ± 1.72aB	1.0 ± 0.26aA	0.20	0.32
Indole butyric acid + calcium chloride	33.1 ± 2.07aA	6.9 ± 1.56aB	0.8 ± 0.29aA	0.18	0.38
As	ns	**	ns	-	-
Plant growth regulator	ns	ns	ns	-	-
As x Plant growth regulator	ns	ns	ns	-	-

Note. Different lowercase letters show significant differences ($P < 0.05$) between plant growth regulators within the same As concentration; different capital letters show significant differences ($P < 0.05$) between with and without As at the same plant growth regulator

regulators. The low root-to-shoot ratio means the plant root was not healthy enough to produce more shoots (Bobeautong et al., 2017). A reduction in the root/shoot ratio has been observed in *Brassica juncea*. It may be due to higher arsenic accumulation in the root as it is in direct contact with the sand, damaging the plant roots (R. Singh et al., 2020). Even though a high concentration of arsenic was observed in the roots of *P. purpureum* cv. Mott receiving indole butyric acid + calcium chloride, the root/shoot ratio of the plant was similar to plants grown in non-spiked soil and receiving the same plant growth regulator formula.

The total chlorophyll contents in the leaves of *P. purpureum* cv. Mott were similar between plants grown under non-spiked soil and arsenic spiked soil. The plant growth regulators had no effects on the total chlorophyll content in the leaves of plants grown under both soil conditions (Table 4). It may be due to the low concentration of arsenic used in this study that did not

Table 4
Chlorophyll content in leaves of Pennisetum purpureum *cv. Mott in the presence of plant growth regulator under non-spiked and arsenic-spiked soil (data shown as mean ± SE)*

Treatment	Chlorophyll *a* content (mg/ml)	Chlorophyll *b* content (mg/ml)	Total chlorophyll content (mg/ml)
Non-spiked soil			
No plant growth regulator	25.2 ± 3.51aA	24.8 ± 5.02aA	50.0 ± 6.59aA
Salicylic acid	22.5 ± 0.27aA	8.8 ± 0.38bA	31.4 ± 0.10aA
Salicylic acid + calcium chloride	29.5 ± 4.35aA	15.2 ± 0.91bA	44.7 ± 5.26aA
Indole butyric acid	31.0 ± 5.58aA	19.6 ± 1.58abA	50.6 ± 4.09aA
Indole butyric acid + calcium chloride	33.0 ± 9.91aA	14.8 ± 3.64bA	47.9 ± 13.39aA
As-spiked soil			
No plant growth regulator	30.0 ± 2.88aA	13.1 ± 1.47aB	43.2 ± 4.31aA
Salicylic acid	20.6 ± 2.94aA	11.1 ± 0.93aA	31.7 ± 3.84aA
Salicylic acid + calcium chloride	20.2 ± 8.21aA	9.5 ± 3.14aB	29.8 ± 11.34aA
Indole butyric acid	19.4 ± 5.05aA	9.7 ± 2.60aB	29.0 ± 7.64aA
Indole butyric acid + calcium chloride	23.8 ± 3.63aA	11.5 ± 2.06aA	35.3 ± 5.65aA

Note. Different lowercase letters show significant differences ($P < 0.05$) between plant growth regulators within the same As concentration; different capital letters show significant differences ($P < 0.05$) between with and without As at the same plant growth regulator

damage the photosynthetic pigment in *P. purpureum* cv. Mott and arsenic was rarely translocated from the root to shoot in this study. A greater arsenic concentration was detected in the roots of *P. purpureum* cv. Mott than in the shoots (Table 5). Only the plant growth regulator and arsenic affected the chlorophyll *b* content in the leaves of the plant. In non-contaminated soil, the chlorophyll *b* content in the leaves of plants receiving all plant growth regulators, except indole butyric acid, was decreased when compared with plants that did not receive plant growth regulators. Arsenic contamination decreased the chlorophyll *b* content in the leaves of plants that received salicylic acid + calcium chloride, indole butyric acid, and did not receive plant growth regulators. However, a reduction in the total chlorophyll content has been observed in *Artemisia annua* after exposure to 100 µM of arsenic, and the application of 100 µM of salicylic acid could increase the total chlorophyll content and biomass of *A. annua* grown under arsenic contamination (Kumari & Pandey-Rai, 2018).

Arsenic Concentration in Soil and Plants

The arsenic concentration used in this study seems to be low to *P. purpureum* cv. Mott, as the plant grew normally in

Table 5
As remaining in soil and plants under various plant growth regulators

Treatment	As remaining in soil (mg/kg)	As in shoot biomass (mg/kg)	As in root biomass (mg/kg)	Bioconcentration factor	Translocation factor	Translocation efficiency (%)
No As	0.81	0.02	0.38	0.49	0.05	7.43
Un plant soil	12.92 ± 0.54a	-	-	-	-	-
No plant growth regulator	11.71 ± 0.48a	3.27 ± 0.27a	19.20	1.92	0.17	17.50
Salicylic acid	11.28 ± 0.30a	2.69 ± 0.12a	10.15	1.14	0.26	26.78
Salicylic acid + calcium chloride	12.70 ± 0.95a	2.50 ± 0.47a	15.03	1.67	0.13	20.02
Indole butyric acid	12.16 ± 0.64a	2.68 ± 0.15a	18.74	1.46	0.18	24.66
Indole butyric acid + calcium chloride	11.07 ± 1.34a	2.70 ± 0.35a	47.38	4.52	0.06	9.77

Note. Different lowercase letters show significant differences ($P < 0.05$) between plant growth regulators within the same As concentration

12.92 ± 0.54 mg/kg of arsenic in the soil. The removal of arsenic from the soil by translocation of arsenic from the soil into the aboveground tissue of the plant in this study was poor because the concentration of arsenic detected in the soil after 41 days of transplantation was not significantly different from the soil without planting (Table 5). Furthermore, there was no significant difference between the arsenic concentrations in the shoot biomass of *P. purpureum* cv. Mott (2.50–3.27 mg/kg) in the experimental pots with and without application of each plant growth regulator (Table 5).

In contrast, the highest arsenic concentration in the roots of *P. purpureum* cv. Mott was detected in plants receiving exogenous indole butyric acid + calcium chloride as a plant growth regulator. It corresponds with the bioconcentration factor of arsenic in plants also observed in Napier grass grown under arsenic-spiked soil and receiving indole butyric acid + calcium chloride. Using indole butyric acid + calcium chloride also limits the translocation of arsenic from the root to the shoot of *P. purpureum* cv. Mott compared to other plant growth regulators. The translocation factor and translocation efficiency of arsenic were only 0.06 and 9.77% when the plant received indole butyric acid + calcium chloride as plant growth regulators (Table 5). In general, the aim of using a plant growth regulator was to decrease the translocation of metal from the root to the shoot of the plant.

For example, exogenous auxin, such as indole-3-acetic acid, can reduce arsenic translocation in rice by lowering the arsenic concentration in the upper leaves and internodes, but higher concentrations of arsenic were detected in the lower leaves and internodes of rice (He et al., 2020). Exogenous application of salicylic acid decreased the arsenic accumulation in the shoot of rice by 27% (A. P. Singh et al., 2017). Exogenous application of calcium chloride also decreased the arsenic uptake by 47% and 21% in the shoot and root of rice seedlings exposed to 1 mM arsenic, respectively (Rahman et al., 2015). In this study, the application of salicylic acid alone or indole butyric acid alone did not stimulate the accumulation of arsenic in the root of *P. purpureum* cv. Mott. Increasing the arsenic accumulation in the plant roots in this study may be influenced by indole butyric acid in combination with calcium chloride. Normally, calcium is an essential mineral nutrient in plants and acts as a secondary messenger that mediates cell and plant development and alleviates arsenic toxicity by reducing its uptake in the plant (Rahman et al., 2015).

Moreover, an appropriate amount of exogenous calcium should be helpful to increase the integrity of the plant membrane (Boorboori et al., 2021). However, applying salicylic acid in combination with calcium chloride did not stimulate the accumulation of arsenic in the root of *P. purpureum* cv. Mott in this study. It may be due to the interaction of salicylic acid and calcium, which has been reported to be involved in

hormonal signal transduction (Medvedev, 2005). The different interactions between calcium with other plant growth regulators, salicylic acid, and indole-3-acetic acid, on arsenic accumulation, may depend on different mechanisms of each plant growth regulator on plant water uptake. Indole-3-acetic acid and calcium ion have been reported to increase water uptake, while salicylic acid has been reported to adjust osmotic pressure and decrease plant transpiration (Khushboo et al., 2018; Saruhan et al., 2012; Votrubová &Votruba, 1986).

The most likely mechanism for arsenic phytoremediation in this study was phytostabilization when considering the amount of arsenic detected in the root and shoot biomass of *P. purpureum* cv. Mott, translocation factor, and translocation efficiency. Successful arsenic phytostabilization was reported for *P. purpureum* cv. Mott when using other organic amendments, such as cow manure and acacia wood-derived biochar (Kowitwiwat & Sampanpanish, 2020). Moreover, ethylenediaminetetraacetic acid (EDTA) was shown to increase the translocation and accumulation of arsenic in the aboveground tissue of *P. purpureum* cv. Mott (Boonmeerati & Sampanpanish, 2021).

CONCLUSION

Successful application of plant growth regulators varied with concentration, type of plant growth regulator, and plant species (Piacentini et al., 2020; Rafiq et al., 2017). The combination of indole butyric acid and calcium chloride was the most suitable for arsenic phytostabilization of *P. purpureum* cv. Mott in this study. However, the application of these plant growth regulators to restrict the arsenic translocation to the aboveground parts of the plant tissue should be further studied under field experiments, especially regarding the effects of other biotic and abiotic factors in the environment.

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UESC	*– Universidade Estadual de Santa Cruz*
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UnB	*– Universidade de Brasília*
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UNISKA	*– Universitas Islam Kalimantan*
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Unsri	*– Universitas Sriwijaya*
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Our goal is to bring high-quality research to the widest possible audience

INSTRUCTIONS TO AUTHORS

(REGULAR ISSUE)

(Manuscript Preparation & Submission Guide)

Revised: December 2020

Please read the *Pertanika* guidelines and follow these instructions carefully. The Chief Executive Editor reserves the right to return manuscripts that are not prepared in accordance with these guidelines.

MANUSCRIPT PREPARATION

Manuscript Types

Pertanika accepts submission of mainly 4 types of manuscripts

- that have not been published elsewhere (including proceedings)
- that are not currently being submitted to other journals

1. Regular article

Regular article is a full-length original empirical investigation, consisting of introduction, methods, results, and discussion. Original research work should present new and significant findings that contribute to the advancement of the research area. *Analysis and Discussion* must be supported with relevant references.

Size: Generally, each manuscript is **not to exceed 6000 words** (excluding the abstract, references, tables, and/or figures)**,** a maximum of **80 references**, and **an abstract of less than 250 words.**

2. Review article

A review article reports a critical evaluation of materials about current research that has already been published by organising, integrating, and evaluating previously published materials. It summarises the status of knowledge and outlines future directions of research within the journal scope. A review article should aim to provide systemic overviews, evaluations, and interpretations of research in a given field. Re-analyses as meta-analysis and systemic reviews are encouraged.

Size: Generally, it is expected **not to exceed 6000 words** (excluding the abstract, references, tables, and/or figures), a maximum of **80 references,** and **an abstract of less than 250 words.**

3. Short communications

Each article should be timely and brief. It is suitable for the publication of significant technical advances and maybe used to:

(a) reports new developments, significant advances and novel aspects of experimental and theoretical methods and techniques which are relevant for scientific investigations within the journal scope;
(b) reports/discuss on significant matters of policy and perspective related to the science of the journal, including 'personal' commentary;
(c) disseminates information and data on topical events of significant scientific and/or social interest within the scope of the journal.

Size: It is limited to **3000 words** and have a maximum of **3 figures and/or tables, from 8 to 20 references, and an abstract length not exceeding 100 words.** The information must be in short but complete form and it is not intended to publish preliminary results or to be a reduced version of a regular paper.

4. Others

Brief reports, case studies, comments, concept papers, letters to the editor, and replies on previously published articles may be considered.

Language Accuracy

Pertanika **emphasises** on the linguistic accuracy of every manuscript published. Articles must be in **English** and they must be competently written and presented in clear and concise grammatical English. Contributors are strongly advised to have the manuscript checked by a colleague with ample experience in writing English manuscripts or a competent English language editor.

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Author(s) **may be required to provide a certificate** confirming that their manuscripts have been adequately edited. **All editing costs must be borne by the authors**.

Linguistically hopeless manuscripts will be rejected straightaway (e.g., when the language is so poor that one cannot be sure of what the authors are really trying to say). This process, taken by authors before submission, will greatly facilitate reviewing, and thus, publication.

MANUSCRIPT FORMAT

The paper should be submitted in **one-column format** with 1.5 line spacing throughout. Authors are advised to use Times New Roman 12-point font and *MS Word* format.

1. Manuscript Structure

The manuscripts, in general, should be organised in the following order:

Page 1: Running title

This page should **only** contain the running title of your paper. The running title is an abbreviated title used as the running head on every page of the manuscript. The running title **should not exceed 60 characters, counting letters and spaces.**

Page 2: Author(s) and Corresponding author's information

General information: This page should contain the **full title** of your paper **not exceeding 25 words**, with the name of all the authors, institutions and corresponding author's name, institution and full address (Street address, telephone number (including extension), handphone number, and e-mail address) for editorial correspondence. **The corresponding author must be clearly indicated with a superscripted asterisk symbol (*).**

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Authors' addresses: Multiple authors with different addresses must indicate their respective addresses separately by superscript numbers.

Tables/figures list: A **list** of the number of **black and white/colour figures and tables** should also be indicated on this page. See ***"5. Figures & Photographs"*** for details.

Example (page 2):

Extraction of High-quality RNA from Metabolite and Pectin Rich Recalcitrant Calyx Tissue of *Hibiscus sabdariffa* L.

Nur Atheeqah-Hamzah, Christina Seok Yien Yong* and Umi Kalsom Yusuf

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List of Table/Figure: Table 1.
Table: 1
Figure 1.

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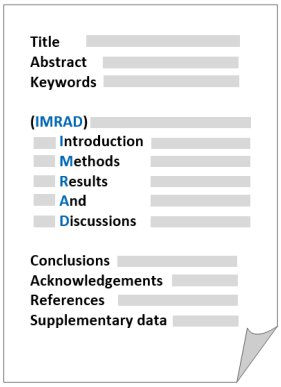
This page should **repeat** the **full title** of your paper with only the ***Abstract***, usually in one paragraph and ***Keywords.***

Keywords: **Not more than 8 keywords in alphabetical order** *must be provided to describe the content of the manuscript.*

Page 4: Text

A regular paper should be prepared with the headings *Introduction, Materials and Methods, Results and Discussions, Conclusions, Acknowledgements, References,* and *Supplementary data* (if any) in this order. The literature review may be part of or separated from the *Introduction*.



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Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words Introduction, Materials and Methods, Results, And, Discussion. It indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: Title, Authors, Keywords, Abstract, Conclusions, and References. Additionally, some papers include Acknowledgments and Appendices.

The Introduction explains the scope and objective of the study in the light of current knowledge on the subject; the Materials and Methods describes how the study was conducted; the Results section reports what was found in the study; and the Discussion section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the Journal's instructions to authors.

2. Levels of Heading

Level of heading	Format
1st	**LEFT, BOLD, UPPERCASE**
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3rd	**Bold, Capitalise each word, ending with .**
4th	***Bold italic, Capitalise each word, ending with .***

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These must be set up clearly and should be typed double-spaced. Numbers identifying equations should be in square brackets and placed on the right margin of the text.

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- All tables should be prepared in a form consistent with recent issues of *Pertanika* and should be numbered consecutively with Roman numerals (Table 1, Table 2).
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  PVY infected Nicotiana tabacum *plants optical density in ELISA*
- Explanatory material should be given in the table legends and footnotes.
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- Authors are advised to keep backup files of all tables.

***** Please submit all tables in Microsoft word format only, because tables submitted as image data cannot be edited for publication and are usually in low-resolution.***

5. Figures & Photographs

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6. Acknowledgement

Any individuals and entities who have contributed to the research should be acknowledged appropriately.

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References begin on their own page and are listed in alphabetical order by the first author's last name. Only references cited within the text should be included. All references should be in 12-point font and double-spaced. If a Digital Object Identifier (DOI) is listed on a print or electronic source, it is required to include the DOI in the reference list. Use Crossref to find a DOI using author and title information.

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	Journals	
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Journal article with 3 or more authors	*For all in-text references, list only the first author's family name and followed by* 'et al.' **Information prominent' (the author's name is within parentheses):** … (Kumar et al., 2020) … … (Kumari et al., 2019) … Or **'Author prominent' (the author's name is outside the parentheses):** Kumar et al. (2020) … Kumari et al. (2019) …	Kumar, A., Padhee, A. K., & Kumar, S. (2020). How Indian agriculture should change after COVID-19. *Food Security*, *12*(4), 837-840. https://doi.org/10.1007/s12571-020-01063-6 Kumari, R., Choudhury, D., Goswami, S., & Dey, N. (2019). Physiological, biochemical, and molecular screening of selected upland rice (Oryza sativa L.) lines from eastern India. Bulletin of the National Research Centre, 43(1), 56. https://doi.org/10.1186/s42269-019-0087-9
Journal article with more than 20	**Information prominent' (the author's name is within parentheses):** … (Tobler et al., 2017) … Or **'Author prominent' (the author's name is outside the parentheses):** Tobler et al. (2017) …	Tobler, R., Rohrlach, A., Soubrier, J., Bover, P., Llamas, B., Tuke, J., Bean, N., Abdullah-Highfold, A., Agius, S., O'Donoghue, A., O'Loughlin, I., Sutton, P., Zilio, F., Walshe, K., Williams, A. N., Turney, C. S. M., Williams, M., Richards, S. M., Mitchell, N. ... Cooper, A. (2017). Aboriginal mitogenomes reveal 50,000 years of regionalism in Australia. Nature, 544(7649), 180-184. https://doi.org/10.1038/nature21416
Journal article with an article number	**Information prominent' (the author's name is within parentheses):** … (Bougnom et al., 2020) … Or **'Author prominent' (the author's name is outside the parentheses):** Bougnom et al. (2020) …	Bougnom, B. P., Thiele-Bruhn, S., Ricci, V., Zongo, C., & Piddock, L. J. V. (2020). Raw wastewater irrigation for urban agriculture in three African cities increases the abundance of transferable antibiotic resistance genes in soil, including those encoding extended spectrum β-lactamases (ESBLs). *Science of The Total Environment*, *698*, 134201. https://doi.org/10.1016/j.scitotenv.2019.134201
Journal article with missing information	**Information prominent' (the author's name is within parentheses):** … (Pryce et al., 2018) … … (Saberi et al., 2018) … … (Rahman et al., 2020) …	**Missing volume number** Pryce, J., Choi, L., Richardson, M., & Malone, D. (2018). Insecticide space spraying for preventing malaria transmission. *Cochrane Database of Systematic Reviews*, (11), CD012689. https://doi.org/10.1002/14651858.CD012689.pub2

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	Newspaper	
Newspaper article – with an author	… (Morales, 2020) ... Or ... Morales (2020) ...	Morales, C. (2020, November 13). Scientists destroyed a nest of murder hornets. Here's what they learned. *The New York Times*. https://www.nytimes.com/2020/11/13/us/murder-hornets-us.html
Newspaper article – without an author	("Japan bird flu outbreak", 2020). OR "Japan bird flu outbreak" (2020) … Use a shortened title (or full title if it is short) in Headline Case enclosed in double quotation marks.	Japan bird flu outbreak spreads to farm in fourth prefecture. (2020, December 01). *The Straits Times*. https://www.straitstimes.com/asia/east-asia/japan-bird-flu-outbreak-spreads-to-farm-in-fourth-prefecture
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Published Dissertation or Thesis References	… (Sutradhar, 2015) ... Or … Sutradhar (2015) ...	Sutradhar, M. (2015). *Metagenomic analysis of rhizospheric microbial diversity in rice grown under irrigated and aerobic condition* [Master's thesis, University of Agricultural Sciences]. KrishiKosh. http://krishikosh.egranth.ac.in/handle/1/5810027635
Unpublished Dissertation or Thesis References	… (Rahman, 2017) ... Or … Rahman (2017) ...	Rahman, F. (2017). *Ecological assessment of the reintroduced milky stork population in Malaysia* [Unpublished Doctoral dissertation]. Universiti Putra Malaysia.
	Conference/Seminar Papers	
Conference proceedings published in a journal	… (Dotaniya & Meena, 2015) … Or Dotaniya and Meena (2015) …	Dotaniya, M. L., & Meena, V. (2015). Rhizosphere effect on nutrient availability in soil and its uptake by plants: A review. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *85*(1), 1-12. https://doi.org/10.1007/s40011-013-0297-0
Conference proceedings published as a book chapter	… (Kurbatova et al., 2019) … Or Kurbatova et al. (2019) …	Kurbatova, S. M., Aisner, L. Y., & Naumkina, V. V. (2019). Some aspects of the essence and legal regulation of agriculture digitalization as one of the priorities of modern state policy of agriculture development. In *IOP conference series: Earth and environmental science* (Vol. 315, No. 3, p. 032021). IOP Publishing. https://doi:10.1088/1755-1315/315/3/032021

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Online	… (Melanie et al., 2017) … Or Melanie et al. (2017) …	Melanie., Rustama, M. M., Kasmara, H., Sejati, S. A., Fitriani, N., & Madihah. (2017, October 25-26). *Pathogenicity of* Helicoverpa armigera *polyhedrosis sub culture virus (*Ha*NPV$_1$) on* Spodoptera litura *Fabricius* [Paper presentation]. Prosiding Seminar Nasional Penelitian dan Pengabdian pada Masyarakat (SnaPP) 2017 Sains dan Teknologi, Bandung, Indonesia. http://proceeding.unisba.ac.id/index.php/sains_teknologi/article/view/988/pdf
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8. General Guidelines

Abbreviations: Define alphabetically, other than abbreviations that can be used without definition. Words or phrases that are abbreviated in the *Introduction* and following text should be written out in full the first time that they appear in the text, with each abbreviated form in parenthesis. Include the common name or scientific name, or both, of animal and plant materials.

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